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RESPIRATION IN THE AFRICAN SWAMPWORM *ALMA EMINI* MICH.

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INTRODUCTION

During a visit to Paraguay in 1926–27 it was noticed that a common glossoscolecid oligochaet *Drilocrius* sp. found in swamp muds was in the habit of exposing to the air the grooved dorsal surface of the hind-end. In view of the highly vascular condition of this surface, and of the probable absence of oxygen from the mud, it was deduced that this was a unique example of a localized organ for aerial respiration in an oligochaet (Carter & Beadle, 1931). The same conclusion was drawn from later observations on *Alma emini*, another glossoscolecid of the same subfamily (Microchaetinae), found in similar situations in the swamps of East Africa (Beadle, 1933).

Since then no further observations were made until a recent opportunity has enabled me to make a more thorough analysis of the respiratory mechanism of *A. emini* in relation to the relevant environmental conditions.

The object of this paper is to give a general account of those aspects of the ecology, behaviour and physiology of this remarkable animal which are concerned with respiration and to indicate the directions in which further research is being done.

THE ENVIRONMENT

(1) *Typical habitat*

The extent of the swamps of East and Central Africa is very great. In Uganda alone there are 2000–3000 sq.miles of permanent swamp and very large areas of seasonal swamp. The predominant plant is *Cyperus papyrus*; over large areas it is the only species, but other species are not uncommon and sometimes *C. papyrus* is absent. Some attention is now being paid to these swamps as a possible reservoir of good agricultural land.

Except at the edges of the swamp the plants are growing in a mat several feet thick of waterlogged and decomposing vegetable matter containing varying amounts of silt, and often floating on water. There is normally little or no exposed surface water, except after heavy rains, apart from occasional shallow puddles, which may disappear in the dry season. The degree of waterlogging varies with the season and with the unevenness of the surface, but for most of the time it is waterlogged to the surface. Over large areas *Alma* is very abundant and the surface is often covered with heaps of fine black mud cast up by them. There can be little doubt that these and other oligochaets must play a part similar to that of the European earthworms in the breakdown of organic matter, though they differ from them in their ability to live in a waterlogged and anaerobic environment. Like peat, the material below the surface is light brown in colour and rapidly darkens by oxidation when exposed to the air. It is composed of plant remains in all stages of decomposition and of living roots of *C. papyrus*. Worms can be found in this to a depth of about 2 ft., and frequently inside decaying pieces of *C. papyrus* stem.

(2) *Oxidation-reduction potentials*

In the present connexion the oxidation-reduction potential of this material is of obvious interest. To measure this a 5 × 2 mm. bright platinum electrode and small glass electrode were clasped together on a screw stand mounted on a wooden board placed firmly on the mat. In this way the two could be lowered slowly and with precision into the waterlogged surface and potentials could be measured from a fraction of a cm. down to about 30 cm. below the surface with the minimum of disturbance. A Cambridge Instrument Co. dip-type calomel electrode was used as reference and the potentiometer was a Marconi battery operated model T.F. 886 placed on the board. Both electrodes were standardized before and after a series of readings, the glass in a suitable buffer, the platinum in a buffered quinhydrone solution giving a standard potential. The latter was always correct to within 7 mV. The potential readings were corrected for temperature and to pH 7 (E_7).

The results will be published more fully in a later paper dealing with other conditions. Here it will be sufficient to record two series of measurements made in sites where *Alma* was abundant. In one of these (Fig. 1 *a*) the water just reached the surface to form a film above the mud, the potential within 2 cm. of the surface was less than +100 mV. and at 30 cm. less than −50 mV., the pH being at all depths between 6 and 7. At another nearby site the surface, though still moist, was just beginning to dry and complete waterlogging began at about 8 cm. (Fig. 1 *b*). The potential near the surface was thus higher (+170 mV. at 2 cm.), but in the waterlogged region below 8 cm. it was as low or lower than at the previous site.

From the work of Pearsall & Mortimer (1939), and of Mortimer (1941) on muds and lake waters in England, it is clear that a potential (E_7) of less than +200 mV. represents an oxygen tension too low to be measured by chemical methods. This has been corroborated for our conditions from parallel measurements of oxygen by Winkler and oxidation-reduction potential on swamp waters. One example is given in Fig. 1 *c*. This was a shallow pool in the surface of the mat where the potential within 0·5 cm. of the surface was less than +200 mV. Winkler estimations (corrected for reducing substances by the sample-blank method) failed to show oxygen in samples taken from as near as possible to the surface.

It can therefore be concluded that the oxygen tension in this waterlogged material is normally too low for aerobic respiration to within a few mm. of the surface. That *Alma* can in fact live indefinitely in mud at extremely low potentials was shown in laboratory experiments in which some were kept in boiling tubes of mud containing methylene blue. On standing for a few days the dye was reduced to within about

0·5 cm. of the surface, the colour immediately returning around an air bubble introduced below the surface or if the upper layer was allowed to dry out and the air to penetrate. Methylene blue is reduced at about zero potential (E_7) and worms will live indefinitely in such tubes of mud maintained in a waterlogged state.

Anaerobic conditions are of course to be expected where bacterial decomposition of vegetable matter is proceeding at temperatures around 28° C., as in the swamp muds near Kampala. There is a considerable production of carbon dioxide as shown by the high tension in the waters of swamp pools (Carter, 1955), and of H_2S which can often be detected by smell in mud pulled up from below the surface. I hope to measure the tensions of these two gases in mud collected in the field in view of their possible influence on respiration.

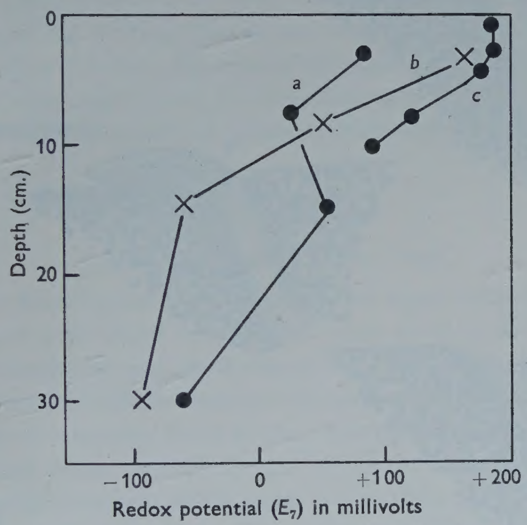

Fig. 1. Oxidation-reduction potentials (E_7) just below the 'mat' surface in dense papyrus swamp. (*a*) Waterlogged to the surface; (*b*) surface still moist but beginning to dry; waterlogged up to about 8 cm.; (*c*) shallow pool of water about 12 cm. deep in surface of mat, containing no detectable oxygen.

(3) *Other habitats*

The type of habitat described above is by far the commonest, but *Alma* is also found in waterlogged soil at the edge of swamps just outside the zone of swamp vegetation. These worms have also been collected from under stones in flowing streams. The oxygen tension here is likely to be high, though no measurements have been made. In the laboratory they have been kept for some days in well aerated water and some have lived for several weeks in a soft 'mud' composed of minced filter-paper in water. There is no doubt, therefore, that they can and do live in well-aerated media.

There is some evidence for an altitude limit to the distribution of *Alma emini* in Uganda. We have failed to find any in the swamps above 5000 ft. in Kigezi and Northern Ankole districts. There is no obvious reason for this other than a lower mean temperature. It is a matter of some possible agricultural importance and should be investigated further.

‘PULMONARY’ RESPIRATION

The most remarkable feature of this animal is its method of breathing air by shaping its hind-end to form a temporary ‘lung’ open to the air at the surface of the waterlogged mud (see Fig. 2). A centimetre* or so of the hind-end is first protruded along the mud surface with dorsal side uppermost. This then becomes spatulate by dorsoventral flattening, the edges being the line of dorsal setae on either side. It may remain in this position indefinitely (Fig. 2B) exposing to the air the grooved dorsal surface of the hind-end. But more often the edges of the groove begin to fold up, like neural folds on a vertebrate embryo, to form a tube opening by a terminal funnel to the air. As the folds close together the worm withdraws its hind-end into the mud (Fig. 2C–E) and, since the cuticle is unwettable, it can retreat even a little below the surface without water running into the funnel (Fig. 2F). In fact, the folds do not always quite meet at the posterior end of the tube, the surface being sufficiently hydrofuge to prevent water getting in between. We have not yet been able to determine the maximum length of this tubular airspace, but it often exceeds

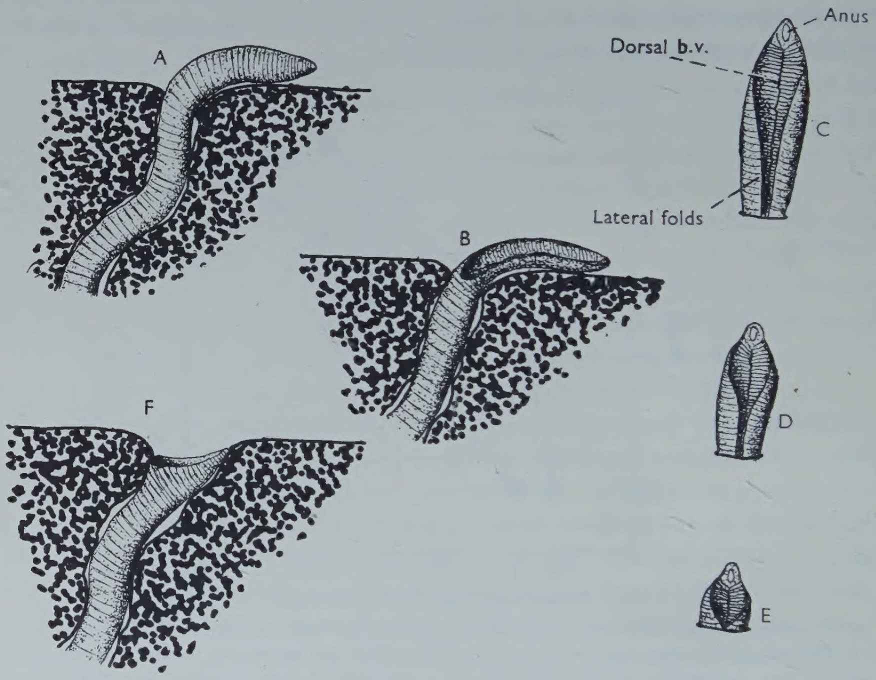

Fig. 2. Exposure to the air of the posterior dorsal surface and formation of ‘lung’. A, Extrusion of hind-end. B, hollowing of dorsal surface. C–E, Dorsal view of extruded hind-end. Stages in the formation and closure of lateral folds and retreat into mud. The dorsal blood vessel and numerous lateral connexions are clearly visible in this region. F, Final position after retreat into mud with tubular lung open to the air.

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* A full-grown specimen is about 30 cm. long.

1 cm. Mr D. P. S. Wasawo, in this laboratory, is investigating the muscular mechanism by which these 'pulmonary' folds are formed.

In the laboratory individual specimens have been seen to remain in this almost submerged position for more than 10 min. A minimum of the body is thus exposed to the attentions of predators such as birds, and the worms will submerge in a flash at the slightest vibration. When kept in dishes with about 1 cm. of water above the mud they would often retreat from the surface with a bubble of air enclosed in the tube. This could not of course be observed in the absence of a superficial layer of clear water, but it is probably a normal and frequent occurrence.

No signs could be seen of any movements which might ventilate the airspace in this tubular lung. Occasionally there were some intermittent but rythmic movements of water from the surface film in and out of the anus. Such have been observed in some other aquatic oligochaets (Stephenson, 1930, p. 190) and it has been suggested that they might assist in respiration or in forcing the blood along the main dorsal vessel. In the present case there is not enough evidence to justify further speculation.

BLOOD SYSTEM

(1) *Vascularization of the 'lung'*

The surface of the dorsal groove which forms the inner wall of the lung is much more richly supplied with blood vessels than other parts of the ectoderm. This is partly due to the immaturity and small size of the segments in this hind-region and the consequent greater number of segmental connecting vessels per unit length of body. But the dorsal ectoderm itself is very heavily invaded by capillaries which lie parallel to and just under the external surface of the ectoderm. These are rare in the lateral and ventral ectoderm of the same region (Fig. 3), and in any part of the ectoderm in the front half of the body. These findings help to justify the conclusion that this is a region of the body surface specially adapted for the absorption of oxygen.

(2) *Properties of the haemoglobin*

The properties and function of the haemoglobin under these peculiar conditions are of obvious interest. A start has been made on an investigation of the oxygen dissociation curve. The method used was a modification of R. Hill's method as used by Fox (1945), whereby the percentage oxygenation is measured spectroscopically, using a Zeiss microspectroscope, against a continuously variable standard human haemoglobin solution. Small but sufficient volumes (about 200 ml.) of the desired gas mixtures were made up manometrically in an apparatus incorporating the graduated gas burette of a Haldane apparatus. In view of the very low tensions involved, the pressure difference due to the introduction of oxygen had to be read with great care. It was also especially necessary to ensure that the nitrogen was absolutely free of oxygen for which purpose it was bubbled through alkaline pyrogallol before passing through a copper furnace.

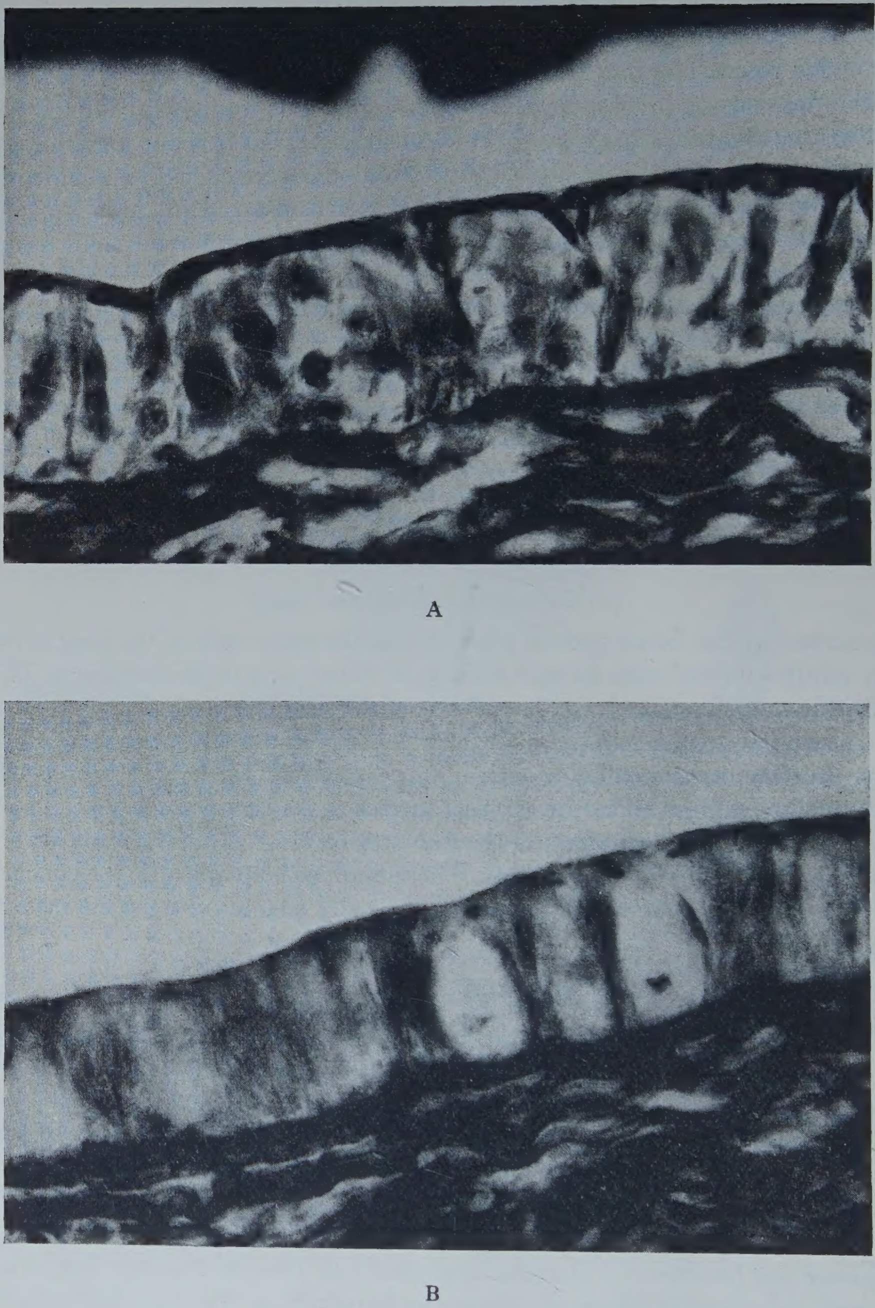

Fig. 3. Transverse sections of *Alma emini* showing vascularization of ectoderm. Stained Mallory plus eosin. $\frac{1}{12}$ in. oil immersion. Preparations by Mr D. P. S. Wasawo. A, dorsal ectoderm near hind-end, showing massed capillaries running just under and parallel to the surface. B, ventral ectoderm of same section.

The blood was obtained by exposing a portion of the dorsal blood vessel of a chlorotone-narcotized worm, removing with filter-paper the body fluid so released and puncturing the vessel. The blood was sucked up in a fine pipette as it emerged. This did not ensure against all contamination with tissue debris and body fluid, but the blood was immediately centrifuged and the supernatant was transferred to another tube and kept in a refrigerator at about − 10° C. Under these conditions the blood kept well for at least a week so long as there had been no contamination with the gut contents or tissues whose enzymes will rapidly break down the haemoglobin. With a bulb-capillary type of equilibrating vessel (Fox, 1945) it was found necessary to dilute the sample about three times with distilled water to give the required optical density in a capillary of the smallest practicable bore. In future work it is hoped to overcome the difficulty found in extracting the blood direct from the vessel and to avoid the need for dilution.

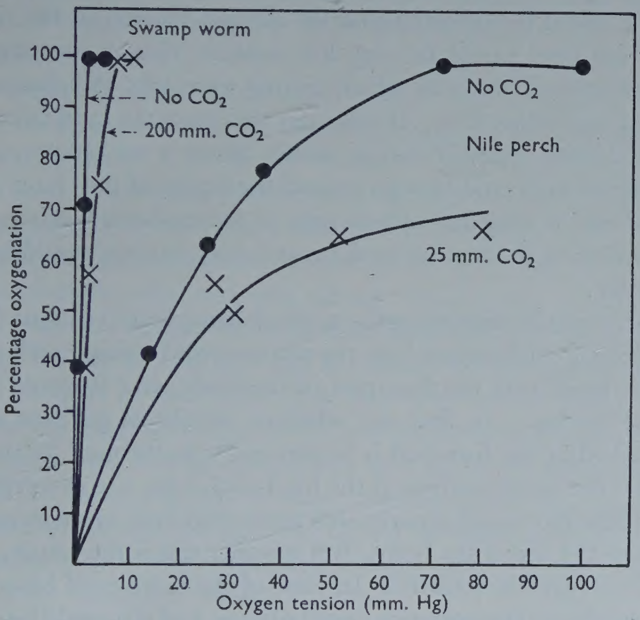

Fig. 4. Oxygen dissociation curves of the haemoglobin of *Alma emini* and the effect of CO_2, compared with the Nile perch (*Lates albertianus*) which inhabits the open well-oxygenated waters of Lake Albert. Curves for *Lates* taken from Fish (1956).

The oxygen dissociation curve of the blood of an animal living in an environment where oxygen is scarce might be expected to be steep and displaced well to the left. In the absence of CO_2 the haemoglobin is in fact saturated at an oxygen tension of 2 mm. Hg (Fig. 4). Apart from this it was the phenomenal insensitivity to CO_2 which was especially striking. It was only when the CO_2 tension was raised to 200 mm. Hg (about 30 % by volume at the altitude of Kampala) that any significant displacement of the curve could be detected. These curves can be compared with those obtained by Fish (1956) using the same apparatus from a haemoglobin solution of about the same optical density taken from the Nile perch, *Lates albertianus*, a fish which lives habitually in open and well-oxygenated water (Fig. 4). The latter has a much lower affinity for oxygen and a very much greater sensitivity to CO_2, the curve being considerably displaced by 25 mm. Hg of CO_2.

DISCUSSION

When breathing air through the hind-end exposed at the surface of the mud, the worm is in rather peculiar circumstances. Assuming that all the body surface is permeable to oxygen, in view of the very low oxidation-reduction potential of the environment, it could be expected that the oxygen tension in the tissues, at least towards the front end, would be very low indeed. This is also suggested by the experiments mentioned above in which worms were kept in tubes of waterlogged mud containing methylene blue. It was clear that even the mud in contact with the worm remained fully reduced except within about a centimetre of the surface, where the oxidized dye could be seen around the region of the 'lung'. On the other hand, the hind-end is supplied with oxygen at atmospheric tension, though in the absence of ventilation the tension at the respiratory surface would be rather lower after a short time.

Krogh (1941, p. 30), in discussing the work of Alsterberg (1922) on *Tubifex* wrote: 'It seems difficult to understand how the worms avoid losing much oxygen by diffusion into the mud from the fore-part of the body, and it would be well worth studying these worms...to find out whether oxygen is perhaps absorbed only through the tail while the fore-part is impermeable to the gas.' With *Alma* there is little doubt that the dorsal surface of the hind-end is the site of oxygen absorption, and the methylene blue-mud experiments prove that little or no oxygen is given off to the mud from the rest of the body. But whether this is the result of a differential permeability to oxygen is doubtful. In view of the scarcity of blood capillaries in the ectoderm except on the posterior dorsal surface, and if it could be shown that the tissues are adapted to respire at extremely low oxygen tensions, it would not be necessary to invoke a differential permeability which *a priori* would appear very unlikely.

The haemoglobin seems well adapted to these working conditions. It can unload at very low tensions of oxygen, and the correspondingly low loading tension might well be an advantage with an unventilated lung and might enable the worm to make some significant use of the bubbles of air which have on occasions been seen to be pulled down below the surface enclosed in the lung (see above). Nearly all the oxygen could be extracted from these by the blood. The extraordinary insensitivity to CO_2 is needed in an environment where the tension is likely to be very high, and it is hoped that we shall be able to determine just how high this tension can be.

In the meantime observations have been made on the effects of high CO_2 tensions on the general behaviour and condition of these animals. It was in fact found that they were apparently quite unaffected over a period of 48 hr. enclosed in sealed bottles of water saturated with pure CO_2. The maintenance of the high tension throughout was checked by addition of a suitable indicator. Though CO_2 is usually toxic, such resistance to high tensions has been recorded with several other animals—e.g. *Tubifex* (Alsterberg, 1922), some soil oligochaets and insect larvae (Kupka & Schaerffenberg, 1947), some freshwater Crustacea and insect larvae (Beadle & Beadle, 1949), and *Gastrophilus* larvae (Levenbook, 1951). With most of the resistant species a high tension of CO_2 causes rapid anaesthesia, but Alsterberg reported that *Tubifex* is only temporarily immobilized, and later resumes its normal movements in CO_2. Levenbook found that the behaviour of *Gastrophilus*, like that of *Alma*, is unaffected by an atmosphere of pure CO_2. This phenomenon is a very mysterious one which we are investigating more thoroughly, and, apart from the problems raised in connexion with the internal regulation of pH, it must be concluded that *Alma* is capable of a purely anaerobic metabolism for long periods. If this is so it would appear that the ingenious method of breathing air is superfluous.

On the other hand, the following observations suggest that they 'strive' to get all the available oxygen when it is scarce. Swampworms were kept in jars of mud covered with 6–10 in. of water and thus too deep for them to reach the surface. So long as the water was aerated, the tail end remained just inside the burrow but began to perform anteriorly directed undulatory movements involving up to 2 cm. of the body. This would draw water over this section of the body, the dorsal surface of which is highly vascular. Some few hours after discontinuing aeration the hind-end began to emerge further and further into the water, the undulation now involving all of the emerged part of the body. This may have been a reaction to the progressively decreasing oxygen tension in the now stagnant water due to consumption by the decomposing mud, and the movements were very similar to those of the well-known *Tubifex*. In some cultures in which decomposition was presumably more rapid, worms finally emerged entirely and lay on the surface of the mud. In such cases the undulatory movements became slower and in general they appeared unhealthy and some ultimately died.

It is not of course necessarily a low oxygen tension which elicits these reactions. It may as well be due to the accumulation of other substances due to anaerobic decomposition. But the result of such movements would normally be to increase the oxygen supply to the animal. Further investigations under controlled conditions using perhaps an artificial cellulose pulp 'mud' should give some interesting results and perhaps a clue concerning the function of aerobic respiration. It is possible that in its normal environment the swampworm is unable to exist entirely on an anaerobic metabolism because the toxic substances thereby produced cannot be disposed of to an external medium which is stagnant, highly reducing and in which there is perhaps already a considerable amount of such substances. Oxygen may in fact be concerned primarily with detoxication rather than with energy production. Experiments with stagnant and circulating media might throw further light on this question.

SUMMARY

1. *Alma emini*, a glossoscolecid oligochaet, is a common inhabitant of the waterlogged and often floating mat of decomposing vegetable matter which forms the substratum of the papyrus swamps of East Africa.

2. Oxidation-reduction potential (E_7) measurements in the field show that to within a fraction of a centimetre of the surface this material is strongly reducing and is presumably devoid of free oxygen.

3. *Alma* lives with most of its body immersed in this material, but for much of the time it exposes to the air a tubular 'lung' formed by the upfolding of the edges of the highly vascular dorsal surface at the hind-end. On retreating from the surface bubbles of air are often carried down enclosed in this tube.

4. The dissociation curve of the haemoglobin is such as might well fit the animal to this mode of life. In the absence of CO_2 it is saturated at about 2 mm. Hg of oxygen.

5. A Bohr effect is not detectable unless the tension of CO_2 is raised to about 200 mm. Hg, and even then it is slight. This would also fit it for respiration in a medium in which very high tensions of CO_2 must occur.

6. In the laboratory it is unaffected by prolonged exposure to an atmosphere of pure CO_2.

7. Though apparently capable in the laboratory of a purely anaerobic metabolism, its behaviour in mud-water cultures suggests that it needs oxygen for survival in its normal environment.

8. The possible functions of aerobic and anaerobic respiration are discussed.

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QUICK AND SLOW CONTRACTIONS IN THE ISOLATED SPHINCTER OF THE SEA ANEMONE, *CALLIACTIS PARASITICA*

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There are two kinds of neuromuscular activity in sea anemones that are full of interest for the comparative physiologist: (1) the conspicuous quick withdrawal movements which can be elicited by any strong stimulus; (2) the almost imperceptible and continuous slow changes of shape and position, incorporated in various feeding and pumping activities, which may be elicited by general or local stimulation of moderate intensity.

The quick withdrawal movements are carried out by different groups of muscles in different species. In *Calliactis parasitica* the marginal sphincter muscle carries out the main movement but tentacles, disk and mesenteries are all involved in the full response. In *Metridium senile* the most powerful movement is the retraction of the disk towards the base by the big longitudinal mesenteric muscles, but the marginal sphincter is also involved in closing up the withdrawing animal. In other anemones the movement usually resembles one or other of these two types, but in most it still awaits a full muscular description.

Pantin's (1935*a*) now classical analysis of the quick response in *Calliactis* with electrical stimulation revealed that a single shock normally has no effect, but a second one following not sooner than about 0·2 sec. and not later than about 2·5 sec. elicits a withdrawal movement. Between these limits the closer the stimuli, the bigger the movement obtained. The precise nature of this facilitating system for controlling the size of the response has so far escaped detection. My attempts to find a chemical 'facilitator' by studying the effects of drugs and anemone extracts (Ross, 1945*a*, *b*, 1952) were not conclusive, although some substances, notably tyramine, had certain interesting effects. Apparently neuromuscular transmission in actinians as in crustaceans, insects and many molluscs does not depend on the cholinergic or adrenergic processes familiar in vertebrates and present in annelids and echinoderms amongst the invertebrates (Bacq, 1947; Fredericq, 1947). Electrophysiological studies have thrown much light on facilitation in Crustacea (Katz, 1949), but no one has succeeded in applying these techniques to the Actinozoa.

The slow movements are carried out by the general circular musculature of the column, by the parietal muscles running longitudinally up all the mesenteries adjacent to the column, by the general longitudinal muscles of the mesenteries and by the radial muscles of the disk and mesenteries.

The analysis of the slow movements in *Calliactis* (Pantin, 1935*b*) and in *Metridium* (Batham & Pantin, 1950*a*, *b*, 1954) revealed another group of phenomena. While the slow movements fitted into the apparently inherent rhythmical activities of the whole animal in a strictly co-ordinated way, they could be elicited by definite stimuli, by food, touch, light, as well as by suitably spaced electrical stimuli. As a rule several stimuli were required to produce a slow movement, but, once begun, it could continue in the absence of further stimulation. Latent periods were very long, from 30 sec. to 1 min. or more.

It seemed from these facts that sea anemones must possess two kinds of muscle, quick and slow, for these two kinds of activity. That the muscles taking part in the quick response might also be able to contract slowly was not obvious at first. This idea occurred to me when studying the effects of chemical treatments on *Metridium* also being stimulated with low-frequency shocks (Ross, 1952). Under these conditions the animal slowly shortens down to about one-third of its extended length. It seemed that the longitudinal retractor muscles of the mesenteries must be involved in such a movement. Batham & Pantin (1954) proved this experimentally when they witnessed slow contractions in excised mesenteric retractors and when they recorded both quick and slow contractions in preparations of *Metridium* sphincter. Yet in order to provide a more general proof that 'quick' muscles can also contract slowly it seemed desirable to examine excised preparations of the sphincter of *Calliactis*. Probably in no other muscle in any actinian is the capacity for quick facilitated contraction more fully developed and it provides, therefore, a severe test for the notion that such muscles also possess the equipment for slow contraction.

METHODS

With minor modifications the methods used by Batham & Pantin (1954) (operating in anaesthesia produced by equal parts of $MgCl_2$ (0·4 M) and sea water) yielded preparations of *Calliactis* which returned to full activity. But longer anaesthesia is desirable in *Calliactis*, even 6–8 hr. is not too long (*Metridium* 2 hr.); and it was often necessary to wait several days before the preparation recovered its full excitability, instead of the few hours which Batham & Pantin (1954) found to be sufficient.

The preparations were made at first by cutting an incomplete ring around the animal just below the margin, leaving a strip of column attached through which to stimulate. The sphincter extends about one-quarter of the way down the column, and it is not difficult to estimate where to make the incision. On the inside the incision goes through the disk as close to the margin as possible, so at first the preparation has part of the disk and many tentacles attached to it. The preparations were then trimmed by snipping off the tentacles and attached pieces of disk and mesenteries. (Later on I found it an advantage to snip off most of the tentacles before the first incision.) The attached piece of column was also trimmed by removing mesenteries and scraping off any other extraneous structures.

At first such preparations produce copious amounts of mucus, and the sea water must be well aerated and changed frequently. They may remain tightly closed for a day or two after their removal from the animal, but eventually they relax, all the raw edges seem to heal up and the production of mucus stops. The sphincter region of such preparations generally assumes the form of an expanded ring lying in graceful loops and festoons, and in this condition it responds to the slightest touch by an immediate quick contraction. With care the preparations will remain alive for 1–2 weeks.

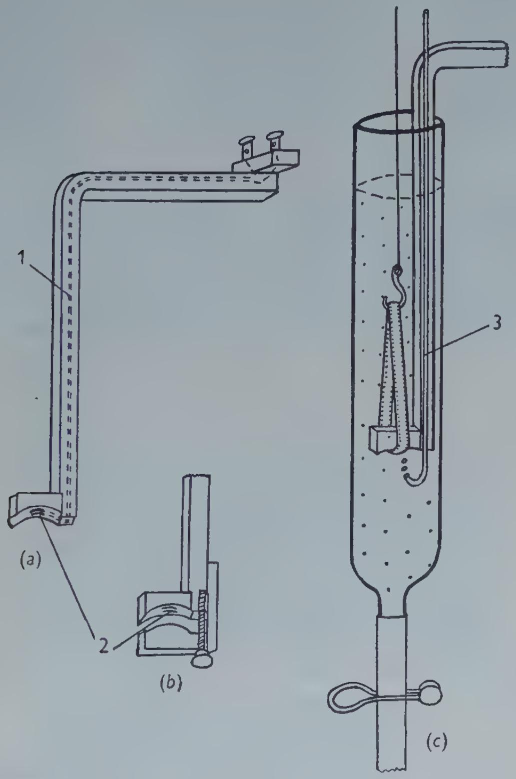


Fig. 1. Perspex (*a*) hook and (*b*) clamp electrodes for stimulating and holding ring preparations in bath (*c*). (1) Electrode leads enclosed in Perspex; (2) exposed platinum electrode tips; (3) air-line to bath.

Preparations made in this way could be pinned out on leaded corks and stimulated through electrodes placed on the attached piece of column. At first I used the Ag/AgCl fluid electrodes which Pantin (1935*a*) recommended to avoid polarization. In later work, where I was studying the effects of various treatments on these preparations and using the smallest possible containers, these fluid electrodes proved very inconvenient, and I tried electrodes with short naked platinum tips. As these did not polarize to any extent, they were used for all later experiments. Also, to assist the study of treatments on the preparation, simple sphincter rings without attached pieces of column were tested and found to be generally satisfactory in that they usually behaved exactly like preparations stimulated through the column. Special Perspex clamps and hooks, carrying platinum wires exposed near the tips, were made to support the preparations in a standard muscle bath (Fig. 1). These provided a simple solution to the problem of keeping the electrodes on the preparation and avoided the tearing that occurs when the preparations have to be fixed down with pins. The results to be presented comprise observations on preparations set up in these various ways, some with pieces of column attached which were stimulated either through fluid-electrodes or through naked platinum-tipped electrodes, and some simple marginal rings stimulated through the Perspex electrode-clamp or hook. The source of the stimuli, as in the earlier work, was a 1 or 2 μF. condenser discharging through a potentiometer to regulate the intensity. Records of contractions were usually made with simple spring levers which allowed some shortening to occur.

RESULTS

General features of the preparation

Preparations of *Calliactis* taken from the marginal region differ in one important respect from the *Metridium* preparations described by Batham and Pantin (1954); they display practically no spontaneous rhythmic activity. An unstimulated preparation may pass many hours attached to a lever writing on a slow drum without showing more than the tiniest changes in length and tension. Yet rings taken from the column at any level below the margin show continuous slow rhythmical contractions like those of *Metridium* preparations, in marked contrast to the inactivity of the marginal region, which is reminiscent of skeletal muscle.

Quick responses

Once the preparations have recovered, usually after 2–3 days, they show the normal frequency-controlled step-like quick response. Fig. 2 shows one series from an experiment at a temperature of 18° C. Clearly it takes exactly the same form as in the intact animal and calls for little comment.

In the vast majority of experiments using simple ring preparations the quick response began only on the second stimulus, but in a few preparations big responses were recorded to leading stimuli or to individual stimuli in a low-frequency series (Fig. 3). It seemed highly probable that on such occasions direct (i.e. non-nervous) contractions of the sphincter were being recorded. This was borne out by tests on preparations with pieces of column attached which happened to display this tendency to respond to single stimuli. In these cases many more quick responses to single stimuli occurred when the electrodes were on the ring than when they were placed on the column. Yet these were exceptional, and I failed completely to find methods of stimulating preparations which would consistently give direct contractions of the sphincter. Even when the electrodes were plunged deep into the sphincter and stimuli of 10 times threshold strength were applied, the response came in on the second stimulus. This is the more surprising since Pantin (1935*a*) reported that a single strong stimulus applied directly above the sphincter gave a direct contraction within 0·03 sec. More work on this aspect of the problem is required since it would greatly assist the further analysis of these neuromuscular activities if a way could be found to stimulate the muscle in the preparations directly, as well as through the nerve net.

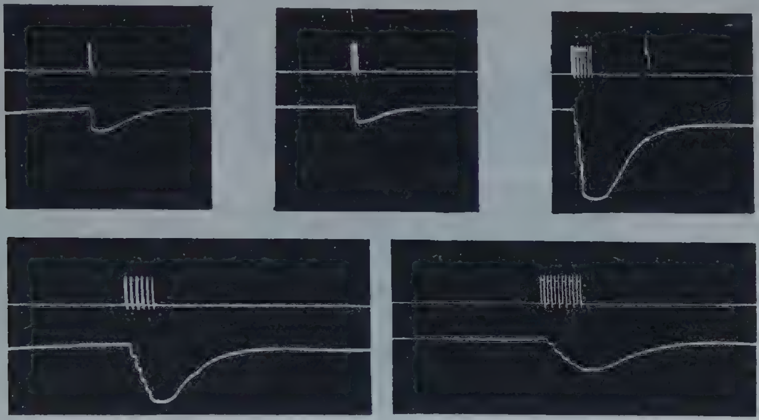


Fig. 2. Facilitated quick responses of *Calliactis* sphincter preparation to 2 stimuli at frequencies of 0·5 and 1·0 sec. and to 6 stimuli at frequencies of 1·0, 1·5 and 2·0 sec. at 18° C.

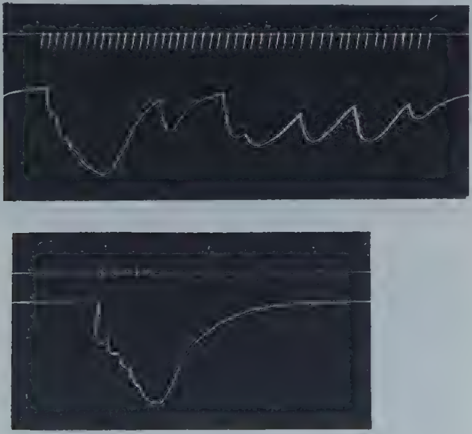


Fig. 3. Quick responses of ring preparation of *Calliactis* sphincter to stimuli at a frequency of 6·0 sec. Presumably responses to direct stimulation of muscle (see text).

Slow responses

When stimuli at intervals of between about 2 and 12 sec. were applied to the sphincter preparations, powerful slow responses were obtained, essentially similar to the slow responses described by Batham & Pantin (1954) in *Metridium*. Unlike the quick response, the beginning of the slow response does not coincide with the arrival of an impulse at the sphincter. It can begin long after the stimuli have ceased and may show latent periods of up to 2 min. Fig. 4 illustrates the general features of the slow contraction and shows that a certain minimal number of stimuli is required to produce a response, and stimuli in excess of this number add to the size of the contraction. It is not a slow form of the facilitated response seen in the quick contractions, though in a sense facilitation occurs in that a number of preliminary stimuli must be given to get a response at all. But once the response begins, further

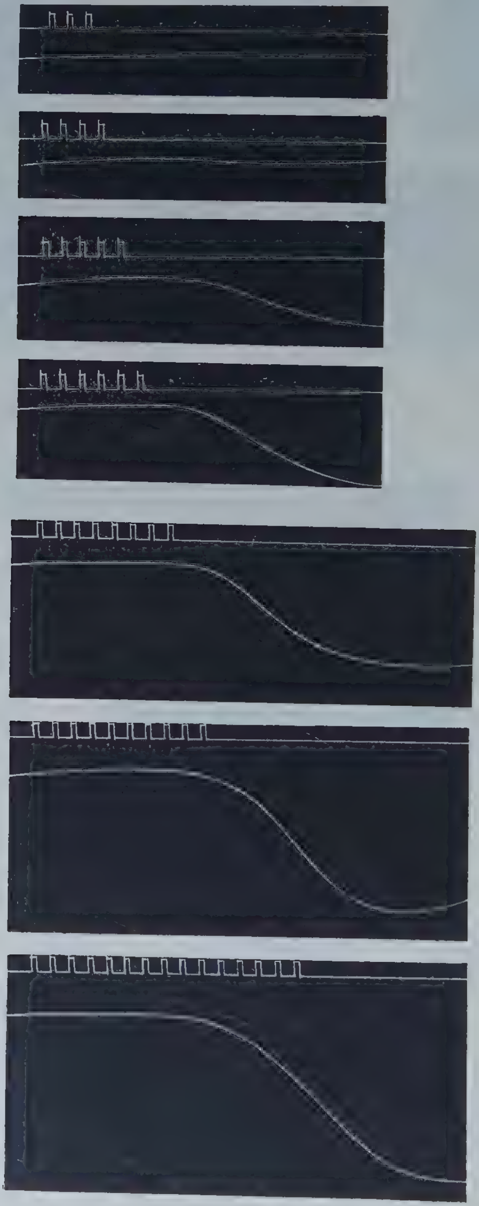


Fig. 4. Slow responses of *Calliactis* sphincter preparation to 3, 4, 5, 6, 8, 10 and 15 stimuli at a frequency of 1 stimulus every 6·0 sec.

shortening with additional stimuli occurs simply by summation. This later phase of the response shows no steps corresponding to individual stimuli in the series and usually gives a smooth sigmoid tracing. Sometimes, however, the movement is uneven and occasionally an additional movement occurs when the stimulation ceases (Fig. 5). Perhaps these latter effects are due to lack of uniformity in the preparation due to faults in operative technique, or to the inclusion in some preparations of slips of muscle which contract when the sphincter is relaxing. Some evidence of the existence of such muscles in the submarginal region will be presented in another paper.

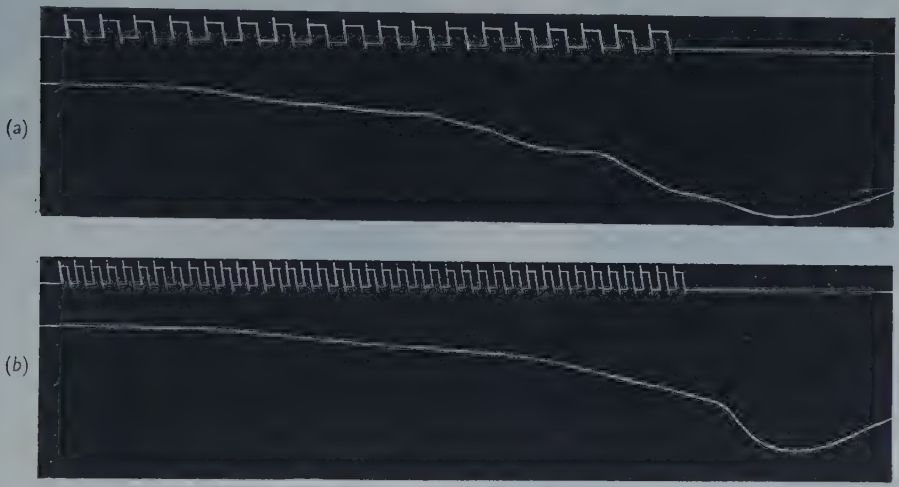


Fig. 5. Atypical slow contractions of *Calliactis* sphincter preparations showing (*a*) uneven response at frequency of 1 stimulus every 9·0 sec., and (*b*) post-stimuli contraction after shocks at frequency of 4·25 sec.

It was obvious from the first experiments that the slow contraction in *Calliactis* sphincter had quantitative features which, although not so exactly determined as those of the facilitated quick response, deserved further investigation. In the sphincter preparation the slow response is very powerful and there is almost no background of rhythmical activity. It therefore offered excellent material for a quantitative study. There are, however, big differences in the size and latent periods of the slow contractions in different preparations, but single preparations kept under good and uniform conditions give consistent performance over several days. Most of the information to be presented on the relationships between size and latent period of the slow contractions, and frequency and number of stimuli, is based, therefore, on a few preparations which were studied intensively.

Fig. 6*a* shows one example in which stimuli were sent in up to the moment when the slow contraction was just beginning. Over the frequency range 3–15 sec. at 19° C., one gets a series of responses rising in height down to a frequency of 6·5 sec., then falling off, until by 15 sec. only a trace remains. It is clear that the excitation process in this contraction has some very definite temporal characteristics.

With a fixed number of stimuli a similar result is obtained. Fig. 6*b* shows a series of slow responses to 5 stimuli at intervals of from 4·8 to 27 sec. at 14° C. Here the biggest response occurred at 13·5 sec., and even at 27 sec. a fair response still occurred. Allowing for the slowing down of processes at the lower temperature, the figures are very much alike. In Fig. 6*b* the quick responses to 5 stimuli at 1·2, 2·4 and 3·6 sec. are included, to show the small slow contractions recorded during the relaxation phase in the same experiment.

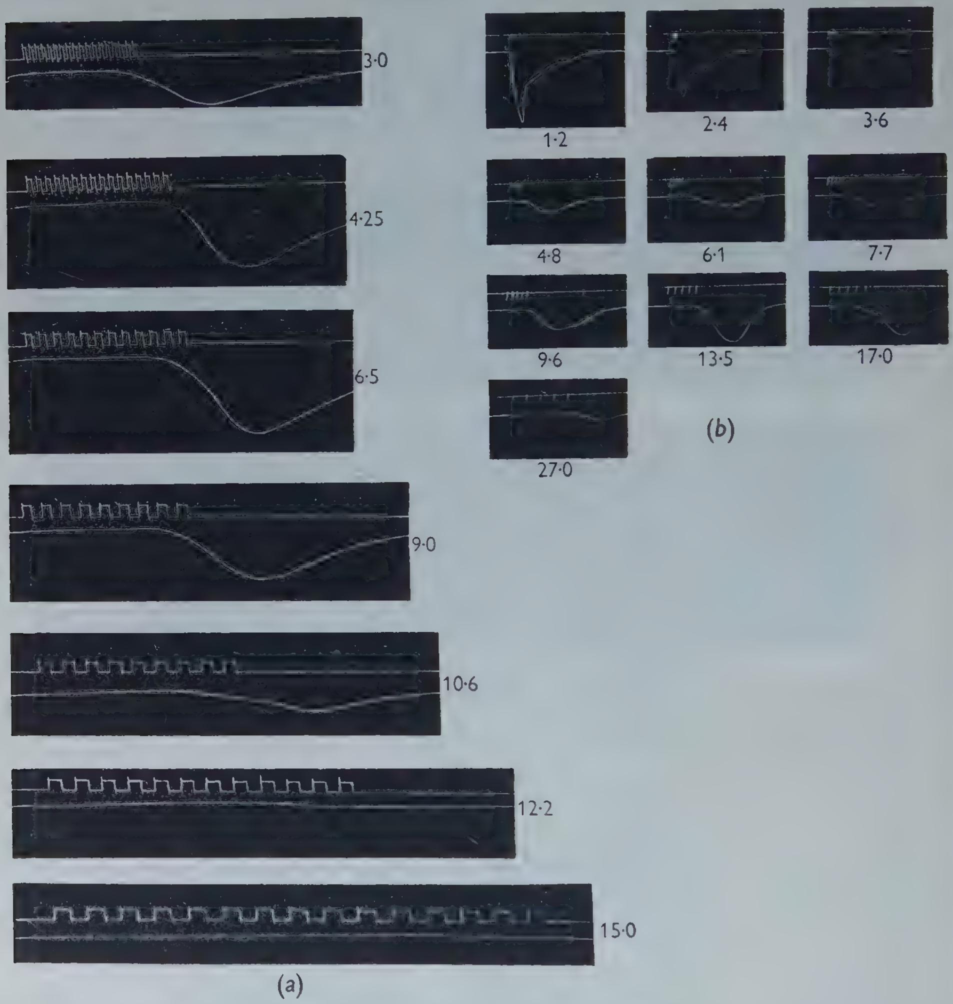


Fig. 6. Slow contractions of *Calliactis* sphincter preparations at different frequencies of stimulation. (*a*) Stimulated until beginning of slow contraction (18° C.); (*b*) 5 stimuli only at frequencies from 1·2 to 27·0 sec (12–13° C.). (At 1·2, 2·4 and 3·6 sec. the slow contraction appears only as an interruption of the relaxation following the quick contraction.)

Fig. 6 also illustrates the relationship between frequency of stimulation and the latent period of the slow contraction. In general, the higher the frequency the sooner the response begins, but in all the experiments there is a wide range of frequencies over which the latent period rises relatively slowly. Table 1 gives data for three experiments, including those illustrated in Fig. 6, both for latent period and size of response over the entire frequency range. It shows latent periods becoming longer at the lower frequencies until the response fails altogether and the latent period becomes infinite.

Table 1. *Latent periods and size of slow contractions at different frequencies of stimulation in three experiments with* Calliactis sphincter *preparations*

Experiment 19. ix. 54, 17–18° C.			Experiment 29. viii. 54, 19° C.			Experiment 18. x. 55, 14° C.		
Frequency (sec.)	Latent period (sec.)	Slow response to 12 stimuli (mm.)	Frequency (sec.)	Latent period (sec.)	Slow response (Fig. 7*a*) (mm.)	Frequency (sec.)	Latent period (sec.)	Slow response to 5 stimuli (mm.)
1·5	*40·4	—	—	—	—	1·2	25·2	2·5
2·0	*44·7	—	2·00	27·6	8·0	2·4	25·2	2·5
2·75	*53·1	—	—	—	—	3·6	28·0	3·0
3·10	57·4	3·5	3·10	44·7	12·5	4·8	30·8	4·0
4·25	63·7	11·0	4·25	55·2	23·5	6·1	39·2	4·0
6·50	68·0	27·5	6·50	57·4	27·0	7·7	39·2	4·0
9·00	95·6	27·0	9·00	59·5	17·5	9·6	42·0	7·0
10·60	121·0	9·5	10·60	59·5	7·5	13·6	47·6	12·5
12·20	144·0	7·0	12·20	97·2	2·0	17·0	50·4	10·0
—	—	—	15·00	150·0	< 1	27·0	120·4	3·0

* In responses to > 12 stimuli.

Fig. 7 shows the relation between number of stimuli and the size of the slow contraction obtained, in an experiment in which the interval was 4·5 sec. throughout. Again, as in Fig. 4, one sees that there is a threshold number of stimuli below which no response occurs. Indeed, one of the most impressive features of the slow contraction for the observer is the regularity with which the response appears after a certain number of stimuli are given, almost as if a counter of some kind were involved. Above this threshold, the response increases with the number of stimuli until a maximum is approached, which is 15–18 stimuli in the experiment shown in Fig. 7.

An attempt to reveal something of the kinetics of the slow contraction by interrupting a series of stimuli is illustrated in Figs. 8 and 9. The results depend to some extent upon the point in the series where the interruption takes place. In the examples in Figs. 8 and 9*b*, a subthreshold number of stimuli was given before the interruption and we see that dropping 3 stimuli extinguished any effect of the preliminary group. The omission of even one stimulus reduced the size of the contraction considerably and with 2 stimuli omitted only a trace remained. But where a supra-threshold number of stimuli was sent in before the interruption, separate responses were obtained from the two groups of stimuli (Fig. 9*a*).

In the experiment shown in Fig. 9*c*, an extra stimulus was given at various intervals before or after a supra-threshold group of 5 stimuli. In general, the shorter the interruption, the bigger the response, except when the preceding stimulus was widely separated (18 sec.) from the main group. Then a double contraction occurred as if the first 2 and the last 4 stimuli were acting independently. But from all these records it is apparent that interrupting a series of stimuli for about 12 sec. (normal temperatures) extinguishes the excitatory process which was building up to cause a slow contraction. This figure corresponds, as one might expect, with the frequency (12–15 sec. interval in Fig. 6*a*) at which the slow contraction disappears.

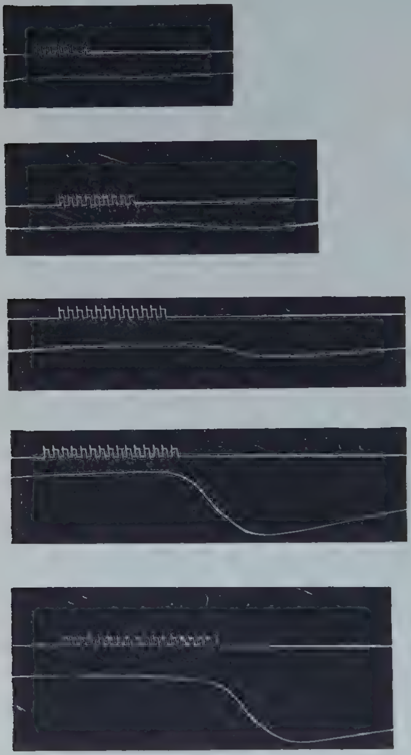


Fig. 7

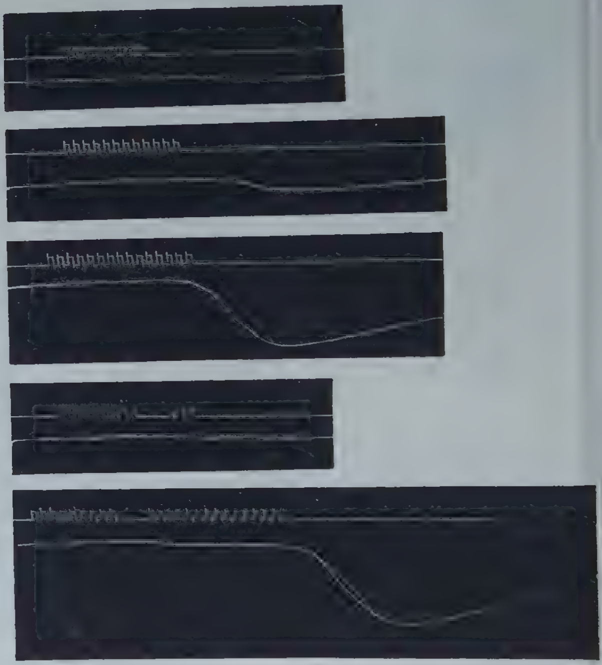


Fig. 8

Fig. 7. Slow contractions of *Calliactis* sphincter preparation to 6, 9, 12, 15 and 18 stimuli at a frequency of 1 stimulus every 4·5 sec.

Fig. 8. Slow responses of *Calliactis* sphincter preparation to 9, 12 and 15 stimuli and to 9+3 and to 9+15 with an interruption of 13·5 sec. in each case. Stimuli at frequency of 1 stimulus every 4·5 sec.

These results show that the slow contraction of *Calliactis* sphincter depends on processes which are fairly exactly determined by the number and frequency of stimuli applied. But the relationship here is not so simple as in the quick response, where the closer the stimuli the bigger the response. In the slow contraction there is an optimal or most economical frequency at which bigger responses are obtained from fewer stimuli. At higher frequencies the slow response begins earlier but it requires more stimuli to start it off, and for any fixed number of stimuli the response will be smaller. Some inferences from these observations will be considered later.

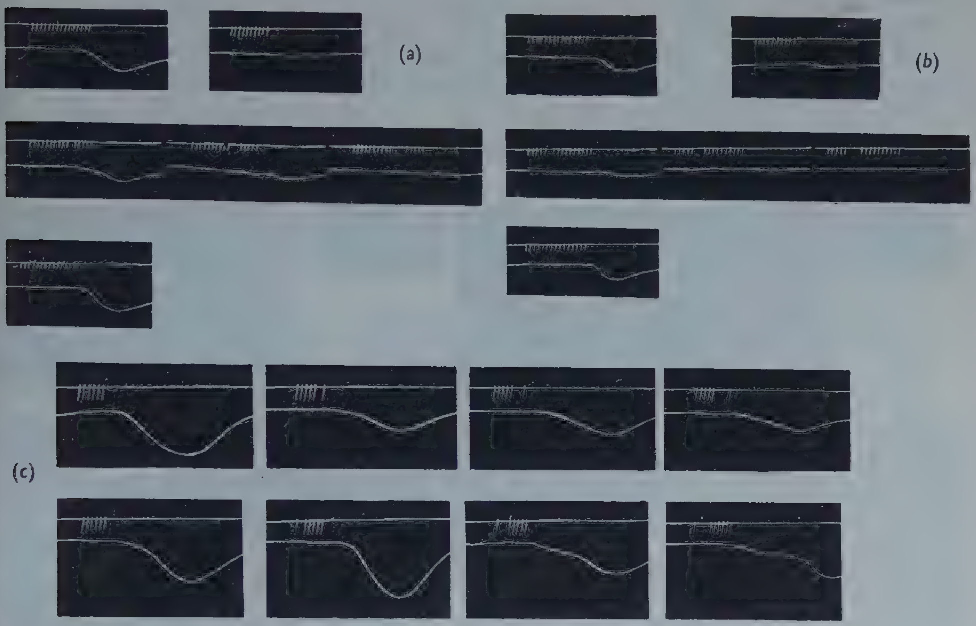


Fig. 9. (*a*), (*b*) Responses to 15 and to 10 stimuli at a frequency of 1 stimulus every 6·1 sec., and (*a*) to 10+5 stimuli and (*b*) to 5+10 stimuli missing out 1, 2 and 3 stimuli between the two groups in each case. (*c*) Responses to 6 stimuli at a frequency of 1 stimulus every 6·1 sec. and to 5+1 stimuli and to 1+5 stimuli missing out 1, 2 and 3 stimuli in each case.

Comparisons between quick and slow contractions

We have seen already that the slow differs from the quick contraction in the non-coincidence of stimulus and response and the requirement of several preliminary stimuli (not just one) before the response can begin. The statement by Batham & Pantin (1954) that 'it is the enormous latent periods which differentiate the slow contractions most completely from the facilitated ones' does not go to the root of the matter in my opinion. The 300-fold difference in latency (0·1 and 30 sec.), remarkable as it is, is not the fundamental difference in the excitatory processes in the two responses. In the quick response, some factor produced by the second stimulus brings in a movement of an explosive kind, provided the facilitating effect of a preceding stimulus has not died away. But essentially the quick contraction is a two-process system requiring separate facilitating and exciting factors. The response beginning on the second stimulus is not due to the summed effects of the two stimuli but to the fulfilment of two requirements, one by the first, and another by the second stimulus. The slow contraction, on the other hand, does not have any of the attributes of a two-process system. It is apparently a straightforward process of accumulating an effect left behind by each stimulus in a series until the total effect reaches a threshold level. Therefore, in spite of the exceptionally long latent period it is a mechanism of a more familiar kind, an example of the summation of subthreshold excitatory events.

Mechanical comparisons between two responses are most instructive. The records illustrated so far were made with simple spring levers which allowed some shortening without permitting the sphincter to close completely. If a lightly weighted isotonic lever is used on the preparation one can see that the slow contraction is no less effective than the quick contraction as a means of closing the sphincter. Fig. 10*a* shows the movements obtained with 2 and 20 stimuli, the interval being 1 sec. The response to 20 stimuli is built up of nineteen separate steps which, although they do not show clearly in a record at this speed, were easily visible to the eye as separate contractions. Yet the sphincter was not completely closed by this movement. Fig. 10*b* shows responses to 5 and 12 stimuli at 2·0 sec. intervals. Small quick contractions were given to every stimulus after the first, but with 12 stimuli, after eleven tiny twitches have shortened the muscle a little, a slow movement sets in which shortens it a good deal more. With stimuli at 4·5 and 6·0 sec. intervals (Fig. 10*c*, *d*), only the slow movement occurred and this produced a smooth contraction which shortened the sphincter almost to its full extent. The slow contraction is therefore able to enlist a contractile mechanism which can completely close the sphincter when it is allowed to do so.

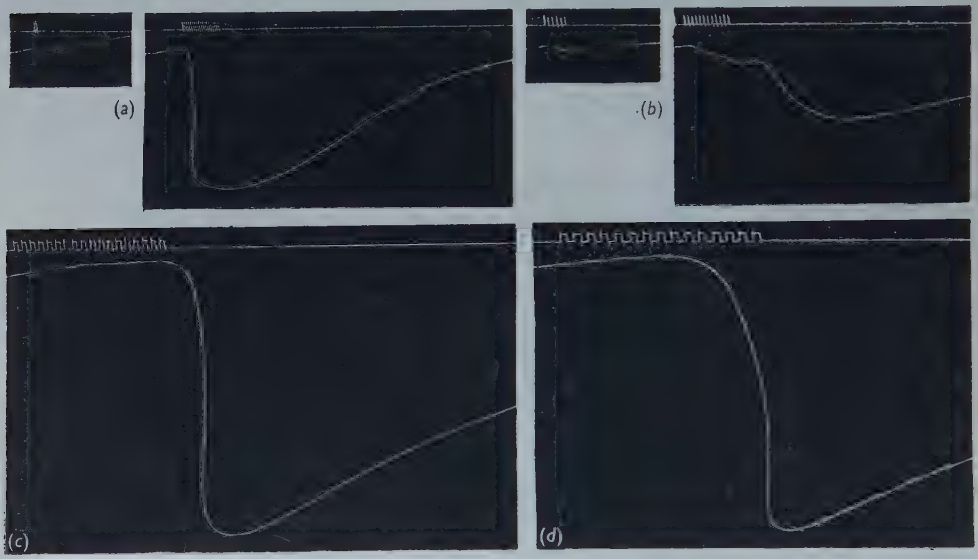
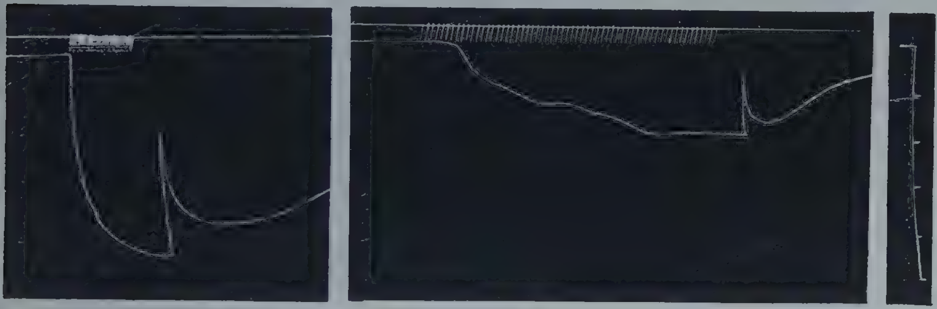


Fig. 10. Records of isotonic contractions of a *Calliactis* sphincter preparation (*a*) to 2 and 20 stimuli, the intervals between stimuli being 1·0 sec., (*b*) to 5 and 12 stimuli, interval 2·0 sec., (*c*) to 18 stimuli, interval 4·5 sec., and (*d*) to 15 stimuli, interval 6·5 sec. In (*a*) only the quick contraction is seen, in (*b*) a series of minute quick contractions on the stimuli is followed by a slow contraction, and in (*c*) and (*d*) only the slow contraction is seen.

If an isometric lever is used which permits very little shortening of the preparation, it becomes plain that the slow contraction is not able to develop the same tensions as the quick contraction. Fig. 11 shows records of the maximal tensions developed in both movements. In these examples, 60–80 stimuli at 1 sec. were required to develop full tension. At the lower frequency of 6·1 sec. about the same number of stimuli, 60–100, was required to develop the maximal tension of an exclusively slow contraction. The tension in the slow contraction never exceeded 50% of the tension produced in a maximal quick contraction. There clearly are mechanical differences between the two contractions in that the slow contraction is unable to enlist the full contractile power of the muscle. This suggests differences in the mode of excitation of the two contractions, a matter that will be discussed later.



(*a*) (*b*)

Fig. 11. Records of isometric contractions in a *Calliactis* sphincter preparation (*a*) to 60 stimuli at a frequency of 1 stimulus every 1·2 sec. and (*b*) to 60 stimuli at a frequency of 1 stimulus every 6·1 sec. with quick release of tension at full contraction in each case.

A second feature of the isometric slow contraction is its uneven development. The bumpiness illustrated in Fig. 11 was a feature of all the isometric records. From the tracing it looks as if the recruitment of the muscle fibres is uneven and perhaps this reflects an uneven spread of excitation through the muscle.

A final observation made on the tension records was the effect of sudden release of tension. In both quick and slow contractions, tension redeveloped very quickly, as shown in Fig. 11, and at about the same rate in the two contractions. This is some indication that there is no fundamental difference between the actual nature of the contractile state in the two movements. In particular, it shows that the slow contraction is not a rigid, tonic, non-elastic state set up in the muscle, but one which has the usual mechanical properties of most muscles under active tension.

In spite of the differences between the quick and slow contractions there are indications that the two contractions do not involve totally separate physiological mechanisms. The same kind of stimuli cause both responses. The thresholds of stimulation are identical. If one stimulates the preparation through a tongue of column attached and gradually severs the attachment, both quick and slow contractions continue to be elicited as long as the tiniest connexion remains. When conduction is finally abolished in this way, presumably by cutting through the last remaining through-conduction tracts, both quick and slow contractions disappear together.

In considering possible relationships between the quick and slow contractions of the *Calliactis* preparation, some attention should be paid to the frequency range over which the two responses overlap (intervals from about 1·5 to 3·0 sec). Here one gets a slow response occurring as a secondary effect following a short staircase (as in Fig. 10*b*). Sometimes, even at higher frequencies, slow contractions appear during the relaxation following a quick contraction to only a few stimuli, but in preparations like that illustrated in Fig. 6*a* the slow response requires so many stimuli to start it off that the huge quick response obscures it at intervals of less than 1·5 sec.

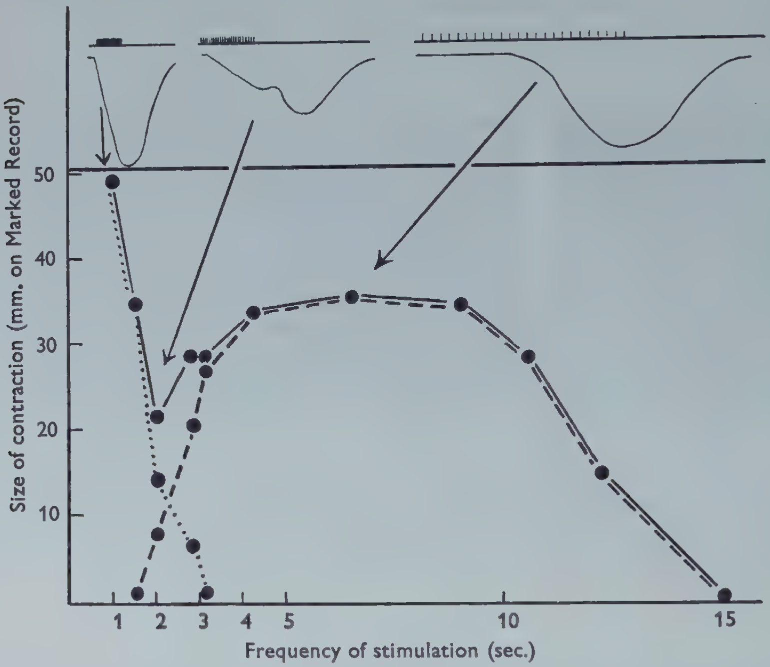


Fig. 12. Size of contraction (quick, slow, and quick + slow) to 24 stimuli over the frequency range of 1 stimulus in 1·0 sec. to 1 stimulus in 15·0 sec. in Exp. 19. ix. 54 A. (17·5° C.). Sketches show form of contraction at points on curve indicated by arrows. Quick contraction, ··· ● ···; slow contraction, - - - ● - - -; quick + slow contraction, —●—.

In the experiments where the effects of frequency and number of stimuli on the size of the response were examined, one can see that the effectiveness of stimuli in producing slow contractions increases as their facilitating effect declines. Indeed, if one plots the results of an experiment in which 24 stimuli were given to a preparation at different frequencies, as in Fig. 12, the slow response is seen to become an effective contractile system at about the point where the quick contraction fades out. This relationship obviously suggests that the slow system makes use of some factor employed in, or produced by, the facilitated quick response system at higher frequencies of excitation.

The discovery of slow contractions in quick muscles like the sphincter of *Calliactis* and the retractors of *Metridium*, means that every stimulus setting up impulses that are transmitted to these muscles leaves two effects behind it. One effect prepares the way, by setting up facilitation, so that a quick response occurs to any stimulus sent in before facilitation decays. The other effect contributes to the build-up of the condition which initiates the slow contraction. The most economical hypothesis would have these two effects, one lasting for 3 sec. and the other for 15 sec. at ordinary temperatures, making different uses of the same basic machinery. One of the tasks of further research will be to compare the properties of the two effects in every possible way to see how they respond to different experimental treatments in order to discover what properties they have in common.

DISCUSSION

The possibility exercised my mind at first that the slow contractions might not be true sphincter contractions but contractions due to the presence of portions of the general slow musculature of the disk and column taken out with the marginal ring. It seems to me now that the almost complete closure of the ring in isotonic contractions (Fig. 10) could hardly be obtained if the contractions were simply due to the fragments of other muscles in the preparations. Furthermore, attempts to divide the preparation in various ways, although not very successful from the standpoint of survival and responsiveness, always produced rings which showed both kinds of contraction. It proved quite impossible to get marginal preparations which displayed quick or slow contractions only. Sections also show the sphincter as a massive undivided muscle and no muscular tissue of any magnitude not recognizable as sphincter appeared in any of the sections of whole animals or preparations that were examined. Moreover, the sphincter is very different from the circular muscle just below it since the latter shows regular rhythmical activity. The absence of such rhythmical activity in the preparations suggests that the general column musculature was scarcely ever included in the marginal rings taken out.

The possible role of the slow contractions of the marginal sphincter in the normal activity of *Calliactis* has formed part of a separate investigation and a full report on this subject will be presented elsewhere. It is enough to state here that slow contractions of the sphincter play a definite part in the rhythmic movements of the whole animal, though complete closure of the margin by the slow system is an infrequent occurrence.

One can do little more than speculate about the physiological properties that make it possible for the sphincter of *Calliactis* to contract in these two ways. The questions that come to mind in this connexion are whether there are two kinds of muscle in the sphincter or whether it is served by two kinds of innervation.

So far there is no reason to believe that there are two kinds of muscle tissue in the sphincter. There are now so many muscles known amongst the invertebrates whose fibres can contract quickly or slowly according to mode of excitation that it would not be surprising to find that all the fibres in *Calliactis* sphincter were similarly equipped. Moreover, Batham & Pantin (1951, 1954) did not find any histological differences between 'slow' and 'fast' muscles, and they saw no buckling during the slow contraction of 'fast' muscles to indicate the existence of an unused fast component in the muscle under these conditions.

It would also be in keeping with the results of studies of quick and slow contractions in the muscles of other invertebrates (Katz, 1949; Hoyle, 1955) if the two activities in *Calliactis* sphincter were served by two different motor pathways. If such differences exist they should appear as two different types of nerve-ending and these should be demonstrable. With the techniques developed by Pantin (1952), which have shown up so much of the detailed nervous organization in *Metridium*, it should be possible to get evidence on this matter.

Isolated preparations offer new possibilities for the study of neuromuscular transmission in these animals. Moreover, the demonstration of the double response of *Calliactis* sphincter heightens interest in the problem of discovering what happens at the junctions in this case. In some ways, the slow contraction, both in the sphincter and in the purely slow muscles, may offer a more fruitful approach to this problem. It appears to be a single-process system and one might expect to find treatments which elicit slow contractions in the absence of stimulation. That the slow response requires a definite number of stimuli to set it off is obviously a fact of some importance in this connexion. While it looks superficially like a counter of some kind at work, it may simply be a matter of latency, the first few stimuli setting off a mechanism which comes into operation after a definite lapse of time. This would be consistent with the double contraction in Fig. 9*c* where the first two stimuli seem to act independently of the remaining four in the series. Such a mechanism would have analogies with the familiar latent periods in responses to light (Hecht, 1918, 1919; Young, 1935; Newth & Ross, 1955). The fact that more frequent stimuli are not equal in effect to ones at lower frequencies (Fig. 6 and Table 1) is open to several interpretations. I have tended to explain this as the accumulation of submaximal effects when the stimuli are closer together. Another type of explanation might be advanced in terms of a long refractory period in the slow-excitor system.

These observations do not throw much light on the problem of the nature of the slow-excitation process and the way it spreads through the muscle. The uneven development of tension in the isometric records might be said to indicate the excitation of different sites, each of which in turn gives rise to a spreading contraction in the neighbourhood.

With the information now available on quick and slow responses it is possible to see some of the comparative aspects of actinian neuromuscular activity much more clearly. Slow rhythmical movements would appear to be the basic primitive form of neuromuscular activity in these animals, and an understanding of the mechanism of slow excitation is therefore fundamental for a general advance of knowledge in this field. The quick response is probably a special adaptation developed to various degrees in different animals and superimposed on the basic slow equipment in muscles specialized to give facilitated quick contractions.

It is very probably true that a satisfactory explanation of the process of neuromuscular transmission in the facilitated quick response will depend on our knowledge of the mechanism of slow excitation. There is probably much to be gained here by extending this kind of investigation to other muscles and other species so that a more comprehensive view of the capacities of actinian nerve and muscle can be presented and perhaps even better material discovered for experimental studies. At any rate it is obvious that this field of investigation, far from being exhausted, is in some respects just beginning to be opened up.

SUMMARY

1. The neuromuscular activities of isolated marginal sphincter preparations of the sea anemone, *Calliactis parasitica*, have been studied. They showed almost no rhythmical or spontaneous activity.

2. In addition to the facilitated step-like quick contractions in response to stimuli at frequencies between 0·2 and 3·0 sec., the preparations gave smooth slow contractions in response to stimuli (usually not less than 6 stimuli were necessary) at frequencies up to about 15 sec. (at 17–18° C.). These contractions are similar to those given by the so-called 'slow' muscles which do not take part in the quick closing movements of the animal.

3. A study was made of the relations between the size and latent period of the slow contraction and the number and frequency of stimuli delivered. At each frequency there is a threshold number of stimuli which only just gives a response; with additional stimuli the response gets bigger until a maximum is reached. There is an optimal frequency (stimuli about 6–8 sec. apart at ordinary temperatures) at which the biggest responses are obtained with fewest stimuli.

4. Certain mechanical and temporal features of the quick and slow contractions are compared and the observations are discussed in relation to current views on neuromuscular transmission and innervation in these and other invertebrates.

My thanks are due to the Director and Staff of the Marine Biological Association's laboratory at Plymouth where much of the work was carried out; to the Royal Society for a grant of £40 from the Browne Fund which enabled me to work at Plymouth; to Dr C. F. A. Pantin, F.R.S., for his unfailing help and good advice at all times; and to Prof. P. B. Medawar, F.R.S., for help in obtaining facilities for the work and for reading the manuscript.

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THE RELATIONSHIP OF OXYGEN CONSUMPTION TO AGE AND WEIGHT DURING THE POST-EMBRYONIC GROWTH OF *LOCUSTA MIGRATORIA* L.

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INTRODUCTION

The novel features of this work on the respiratory metabolism of the growing locust are (1) that successive measurements are made on individual locusts and (2) there is no period of enforced starvation preceding the measurements on oxygen consumption. The first point enables the changes of absolute values of weight and oxygen consumption with age to be considered as no allowance need be made for population variation, the measurements being linked together by being made on the same individual. For this reason, too, the relationship between weight and oxygen consumption can be considered without first of all having to express oxygen consumption as some function of weight (Weymouth *et. alii* 1944). The second point implies that the normal growth of the animal is uninterrupted by enforced starvation, hence the oxygen consumption of the growing animal rather than that approximating to a basal metabolism is being studied.

The disadvantages of these methods are that only a few individuals from each instar can be studied, hence statistics for population values are founded on small samples; also that the individuals studied may not be 'normal' locusts. Some discussion on this latter point will be given after the results have been presented.

THE MATERIAL AND THE ENVIRONMENTAL CONDITIONS FOR GROWTH

The insects for study are females of *Locusta migratoria migratorioides* (R. & F.). Populations of both sexes are supplied by the Anti-Locust Research Centre, London.

The stock and the experimental animals are kept in an insectarium at a temperature of 28° C. and a relative humidity of 70%. Abundant food is always available to them. The lights are controlled to give alternate periods of 12 hr. light and 12 hr. dark with an intensity of 17 foot-candles on the floor of the cages. The presence of these lights causes the temperature of the room to vary from 30° C. during the day to 28° C. at night. Measurements in the cages show that they closely follow the room in temperature, but the introduction of fresh food each morning causes the R.H. to rise to 80% and provides a region of high humidity (90%) into which the locusts can move.

THE SAMPLING OF THE STOCK POPULATION

The stock population is prepared for sampling by removing from it all locusts older than the penultimate instar required for study. The population is then examined at frequent intervals, and those insects that have moulted to the required instar are removed at each observation. The time of ecdysis must lie between the time the animal is removed and that of the previous observation. This interval is referred to as the initial ecdysial range, and its mid-point taken as the time of the initial ecdysis. The newly moulted locusts are sexed (Dirsh, 1950) and the females used in the experiments.

Each individual female is studied throughout the stadium and for one observation at the beginning of the subsequent instar. The period in which measurements are to be made is restricted to this duration for two reasons: (1) the handling of individuals at frequent intervals over long periods of time produces abnormalities in their growth; (2) the possibility of phase change (Faure, 1932) and its effects on respiratory metabolism (Butler & Innes, 1936) is minimized. The isolated females show no evidence of phase change at the commencement of the subsequent instar. Tests were carried out to see if weight and oxygen consumption are changed when the locusts are reared at different population densities. An analysis of four experiments in which weight and oxygen consumption were measured on individuals taken from different population densities, at intervals throughout the stadium and at the commencement of the next instar, failed to show any significant difference in these qualities. It is therefore concluded that any effect of phase change on the growth of the locusts studied may be ignored. Population densities studied range from 1 to 20 per cage of 21 sq. cm. floor area and 450 c.c. volume.

THE DETERMINATION OF AGE DURING A STADIUM

The observations to be made on each individual are commenced as soon after the locust is removed from the stock as is possible. The age of the animal at this observation is taken as being equal to the difference in hours between the mid-point of the initial ecdysial range and the time the observation is made. Thereafter measurements are made at precisely known intervals of time, the series terminating with the one made just before the final ecdysis. From this set of measurements trend lines may be calculated to express the changes undergone during a stadium. Thus the time data on which these trend lines are based are precise, but the relationship between these data and the times of the initial and final ecdysis will be uncertain and the age of the animal at any one observation will not be accurately known. It will not be wrong by a value greater than half the initial ecdysial range. Similarly, the trend-line data can be related to the final ecdysis, the error being not greater than half the final ecdysial range.

Measurements are made at approximately 12-hourly intervals for the first five instars and at 24-hourly intervals for the adult. In every case the duration of the interval between one observation and the next is exactly known, and no approximation is made in the calculations of the trend lines based on them.

THE TECHNIQUE FOR THE MEASUREMENT OF OXYGEN CONSUMPTION

The oxygen consumption of the locust is measured by the direct method of carbon dioxide absorption with potassium hydroxide using the constant-volume type of respirometer of Warburg. The measurements are made at 28 ± 0·01° C. The procedure is based upon the standard technique as described by Dixon (1943) with the following modifications introduced to meet the special needs of the material. Shaking of the manometer during the experiment is omitted, as trial measurements show that this omission does not alter the rate of carbon dioxide absorption by the caustic potash. The volume of the locust is large compared with the amount of air the flask can contain, so that the amount of air available to the animal (V_g of Dixon, 1943) must be measured afresh at each experiment. The volume of gas in the flask which is at atmospheric pressure can have its volume and pressure altered by changing the level of the manometer fluid. Since the capillary of the manometer limb is calibrated in the usual way, the amount of change, volume in mm.^{3} and pressure in mm.^{3} of Brodie's solution is known. By the application of Boyle's law the initial volume of air in the flask is calculated from these measurements. I am indebted to Mr A. Aitken for the use of this method. With the value for V_g known, the amount of oxygen used by each locust at each experiment in mm.^{3}/hour at N.T.P. is calculated from the formula given by Dixon (1943), except that his expression for the volume of fluid in the flask is ignored as being irrelevant in this case.

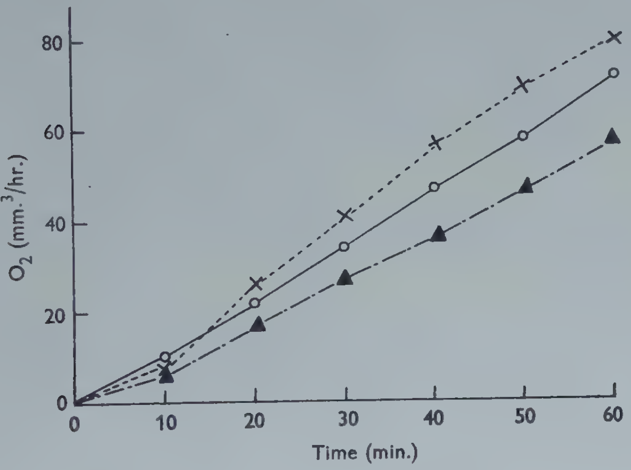

Fig. 1. The oxygen consumption for three individual locusts for a 60 min. period.

During its life the locust undergoes considerable changes in size which necessitates using flasks of different volume for the different sizes. Based on preliminary measurements flasks are chosen of such a size to ensure that the animal does not use up more than 5 % of the available oxygen during the 90 min. it is in the manometer.

On each occasion the manometer is read at 10 min. intervals for the period of 1 hr. These readings are plotted against time to check that the course of oxygen uptake over the hour is linear. Examples of these readings are given in Fig. 1. These show that constant rates of uptake occur, indicating that the technique used is satisfactory.

RESULTS

The results obtained consist of a series of observations on the changes in weight and oxygen consumption an individual locust undergoes during a stadium, and before and after its subsequent ecdysis. Six individuals have been studied for each of the first five instars and for the growing period of the adult stadium. An example is illustrated in Fig. 2 for an individual of the third instar. This graph is quite typical of those obtained for other individuals, showing neither the greatest nor the least amount of oscillation observed.

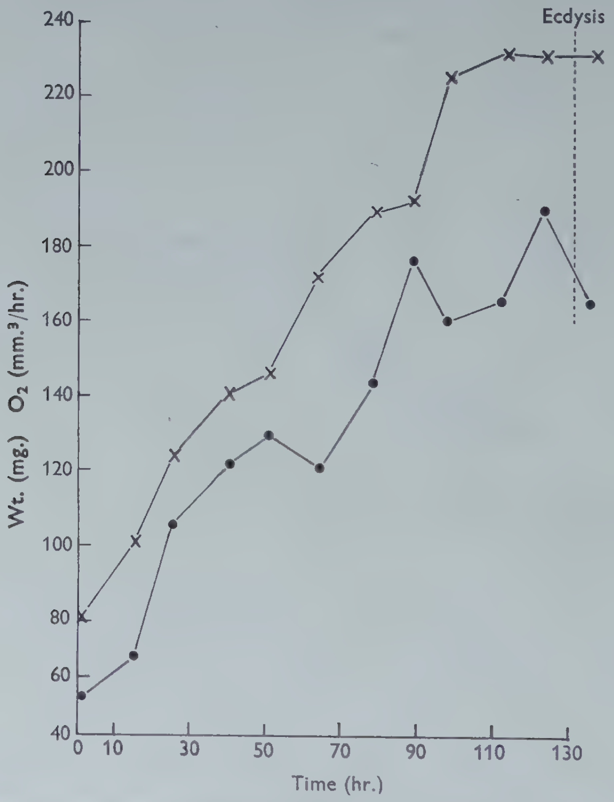

Fig. 2. The changes of weight and oxygen consumption of an individual during the third stadium. Zero time marks the estimated time of the initial ecdysis, and the word 'ecdysis' that of the final ecdysis. × = weight (mg.), ● = oxygen consumption (mm.^{3}/hr.).

Further examination of the data is concerned with the changes of weight and oxygen consumption with age, and with the relationship between changes of weight and changes of oxygen consumption. These two aspects are studied at three levels of time: (1) changes occurring in periods of less than 36 hr. duration, (2) changes occurring over the duration of a stadium, (3) changes occurring over the whole of the growing period of the locust's life. Each of these levels requires a different treatment of the results. The short-period changes are concerned with the values observed of weight in milligrams and oxygen consumption in mm.^{3}/hr. and are linked together by reason of their being made on the same individual.

When weight and oxygen consumption are plotted against time over the complete duration of a stadium straight lines can be fitted to the data. These straight lines can be represented by the general formula,

$$y = a + b\,(x - x'),$$

where y is the value of the quality (weight or oxygen consumption), x is the time, x' is the mid-point of the stadium in time, and a and b are constants which are calculated by the method of least squares for each quality and for each individual. In certain special cases to be discussed later two straight lines are necessary to get a reasonable fit of the trend line to the observed values. In these cases the changes in slope are fairly abrupt and division of data for the calculations is made from an examination of the graphs.

From these trend lines a curve showing the changes of weight and oxygen consumption that occur during the growing period of the locust's life is constructed.

(*a*) *The short-period changes*

In any one individual the pattern of the actual values for weight and oxygen consumption during a stadium shows the irregular manner in which these qualities increase (Fig. 3). A comparison of the pattern of oxygen consumption with that for weight shows that increments or decrements of the former are usually accompanied by similar changes in the latter (Fig. 3), though quite often the reverse is true.

In the presence of abundant food and under optimum environmental conditions the fluctuations of live weight are thought to be consequent upon the normal feeding behaviour of the locust, which consists of periods of eating separated by periods of 'resting'. These irregularities in weight increase are accepted as one of the uncontrollable variables in the growth of the locust and the limits of their magnitude compatible with normal growth must be determined. Certain changes in weight such as decrements due to defaecation or increments due to the taking of a mouthful of food occur so quickly that they might be regarded as instantaneous. For example, the act of defaecation takes less than 5 sec. and can bring about a loss of weight equal to 1·2% that of the animal. The other changes in weight take appreciably longer to occur and a time factor must be given along with the magnitude change. Observations under the conditions in which these locusts are reared show that periods of fasting of 12 hr. and of steady eating for 3 hr. occur sufficiently frequently to be considered as part of the normal behaviour of the animal.

The loss of weight due to a 12 hr. period of fasting is 9 ± 3·3% of the initial weight of the locust. The percentage loss of weight is comparable for all stages and the figure given is the mean for seventy-two individuals, twelve from each instar. Similarly, the increase of weight due to 3 hr. eating is 15 ± 6·3 % of the initial weight of the locust. These values apply to locusts taken from stock which will be in a moderate nutritional state. If the animals have fasted for 12 hr. and are then placed with abundant food an increase of 26 ± 4·2 % of the animal's original weight in 60 min. occurs.

These observations suggest that losses of 12·3 % (mean + standard error) in 12 hr. and gains of 21·3 % in 3 hr. may be expected in a normally growing locust. The irregularities of weight increase of the animals studied here do not exceed these limits, and it is held that growth is normal regardless of whether its rate is fast or slow or the amount great or small.

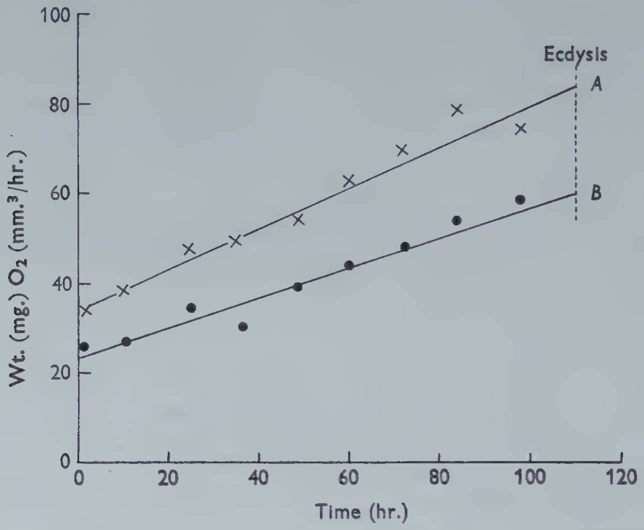

Fig. 3. *A*, the trend for weight and *B*, the trend for oxygen consumption throughout a stadium for an individual of the second instar. Formula for the trend line is $y = a + b\ (x - x')$. In this individual: for weight, $a = 53·8$, $b = 0·4554$; for oxygen consumption, $a = 39·02$, $b = 0·3325$; for both $x' = 47·93$. Actual values at each observation, × = weight (mg.), ● = oxygen consumption (mm.³/hr.).

The change in oxygen consumption for a unit change in weight due to fasting or feeding is determined. This change is found to be comparable in all stages and is −2·899 ± 1·01 mm.³/ hr. for each milligram of weight lost for a 12 hr. period (eighteen individuals measured, six from each of the second, fourth and adult instars). Similarly the change for a milligram of weight increase is + 1·319 ± 0·72 mm.³/hr.

These figures are used to set limits to the range of oxygen consumption that can be regarded as normal for any given weight. Due to the irregularities of weight increase a sample of growing locusts of the same instar and of the same weight will include animals whose feeding histories, immediately prior to the sample being taken, are diverse. For example, the trend line values for weight and oxygen consumption suggest that a locust weighing 100 mg. will have an oxygen consumption of 100 mm.^{3}/hr. But, if the weight is a 'low' due to the animal having fasted for the 12 hr. preceding the measurement, the oxygen consumption will fall below this level. If the weight has fallen from 109 to 100 mg. (approximately 9%), then the oxygen consumption will also fall from 108 to 82 mm.^{3}/hr., and may fall to 73 mm.^{3}/hr. (mean value of the decrease in oxygen consumption plus standard error). If, on the other hand, the weight is a 'high' due to an animal weighing 85 mg. eating for some hours prior to the observation and thus raising its weight to 100 mg. (approximately 15%), the oxygen consumption will increase from 85·5 to 105·3 mm.^{3}/hr. and may rise to 115·9 mm.^{3}/hr. The oxygen consumption of seventeen individuals whose weights lie between 96 and 104 mg. was measured

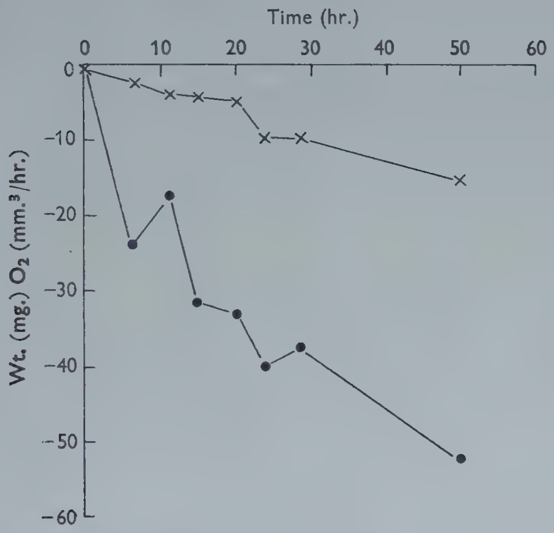

Fig. 4. Loss of weight and decrease in oxygen consumption in a starving locust of the second instar. Measurements made when starvation is commenced are taken as zero and the difference between this and subsequent measurements are plotted above. × = weight (mg.), ● = oxygen consumption (mm. ^{3}/hr.) No allowance is made for the probable time lag between the commencement of starvation and the start of the fall of oxygen consumption.

and was found to range from 71·3 to 122·9 mm.^{3}/hr., which agrees fairly well with the ranges calculated on the above assumptions. The range of oxygen consumption is not further limited by specifying the age of the animal as a sample characteristic since the feeding cycle is independent of age. This situation is also true for the individual which, having the same weight on successive occasions, may show an equally wide range in its oxygen consumption.

The oxygen consumption of the locust does not begin to fall off for at least 1½ hr. after the animal stops feeding, although there is often a decrease of weight due to defaecation during this time. If, however, the loss of weight continues there is a considerable drop in oxygen consumption after 5 hr. (Fig. 4). Similarly, the oxygen consumption does not increase the moment the locust begins to eat. This is most clearly shown in animals that have been fasting (Fig. 5). This shows the changes in oxygen consumption in an individual of the fourth instar following a meal given after 50 hr. starvation. Five other individuals of the fourth instar and six individuals of the second instar show an exactly similar response. From the figure it will be seen that oxygen consumption continues to fall during the 4 hr. period that the locust was feeding, but shows a considerable increase 5½ hr. after the meal has commenced.

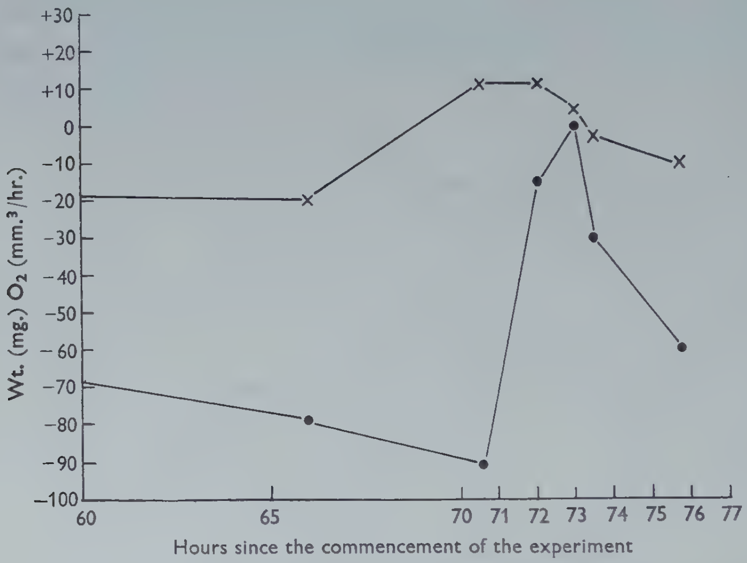

Fig. 5. The effect of a meal on oxygen consumption in a starving locust of the fourth instar. Time is in hours since the animal was isolated from its fellows; weight and oxygen consumption plotted as the differences between the first and subsequent observations. For further explanation see text. × = weight (mg.), ● = oxygen consumption (mm.^{3}/hr.).

(*b*) *Changes occurring during a stadium*

The trend of weight is represented by a straight line which expresses it quite well, although this is not the case for data given for other species of insects by previous workers (Yagi, 1926; Calvert, 1929; Teissier, 1931). The differences are due to: (1) a loss of weight due to emptying the gut which occurs at other times during the stadium as well as just before the ecdysis; (2) moulting, which takes place in suitable environmental conditions very soon after the locust has emptied its gut which it can do in 9 hr. (Voskresenskaja, 1936).

In the adult the weight trend is represented by two straight lines, one for the growing period and one for the period of steady weight (Fig. 6). This abrupt change occupying only 2% of the animal's life is masked in population studies (Davenport, 1931) but is clearly shown in all individuals studied.

The trend for oxygen consumption during a stadium is well represented by a single straight line for instars 1–4 and for the adult, but for the fifth instar the matter is more complicated.

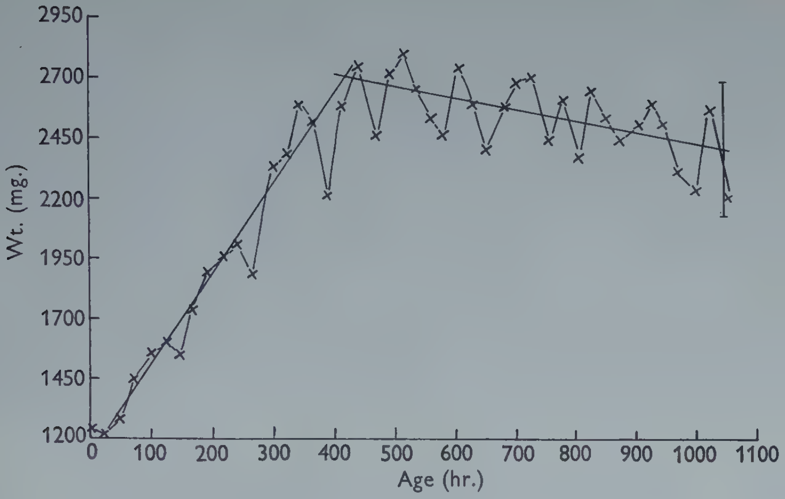

Fig. 6. Changes of weight in an individual during the adult stadium. Trend line values for the formula $y=a+b(x-x')$, for the growing period $a=1938$, $b=3{\cdot}955$, $x'=208{\cdot}5$.

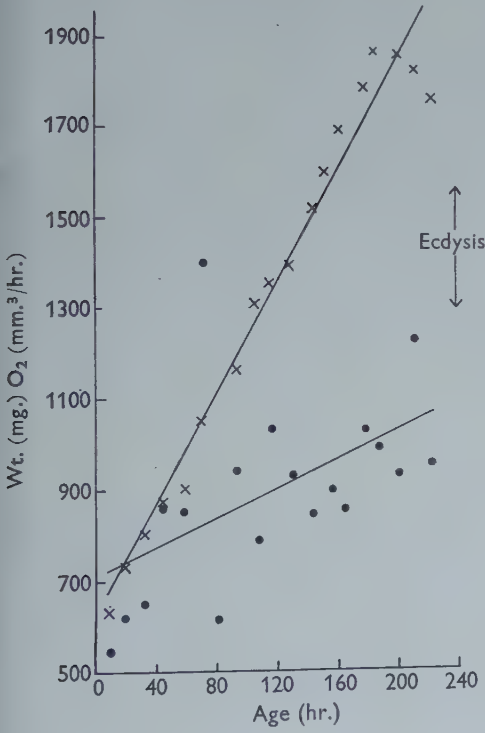

Fig. 7

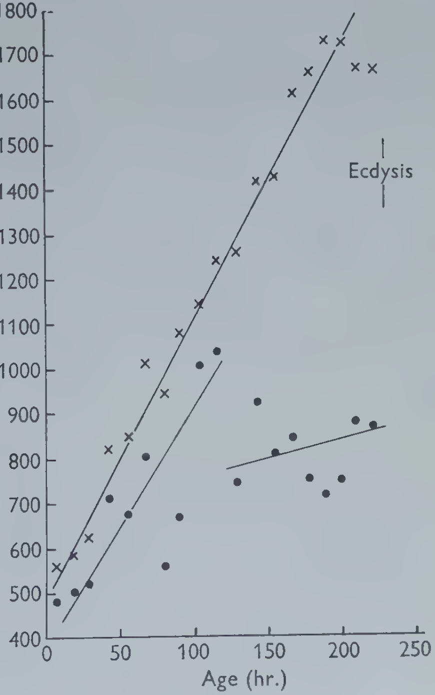

Fig. 8

Fig. 7. Change of weight and oxygen consumption during the fifth instar. Group A. × = weight (mg.), ● = oxygen consumption (mm³/hr.). Values for the formula $y=a+b\ (x-x')$, for weight $a=1312{\cdot}6$, $b=6{\cdot}0804$; for oxygen consumption $a=894{\cdot}1$, $b=1{\cdot}6305$, for both $x'=151{\cdot}58$.

Fig. 8. Change of weight and oxygen consumption during the fifth instar. Group B. × = weight (mg.), ● = oxygen consumption (mm.³/hr.). Values for the formula $y=a+b\ (x-x')$, for weight throughout the stadium $a=1170{\cdot}7$, $b=5{\cdot}5698$, $x'=111{\cdot}50$; for oxygen consumption Part 1, $a=711{\cdot}8$, $b=5{\cdot}7148$, $x'=61{\cdot}58$; Part 2, $a=829{\cdot}7$, $b=1{\cdot}2551$, $x'=175{\cdot}55$.

Individuals of the fifth instar can be divided into two groups with regard to their trends in oxygen consumption, although in the weight trend both groups are similar. Group A contains two individuals in which the trend is adequately represented by a single straight line (Fig. 7). Group B has four members in which the slope of the line changes after about half the stadium has elapsed so that two straight lines are required to fit the observations (Fig. 8). This second group shows that a change in the trend of oxygen consumption can occur whilst the weight of the animal is steadily increasing and at a time not coinciding with an ecdysis.

The co-relationship between trend lines for weight and oxygen consumption is estimated graphically (Tippet, 1952). In the first instar there is clearly a high degree of co-relationship between the trend lines This gradually lessens in successive instars until in the growing period of the adult stadium it is zero. It is important to realize that this only applies to series of measurements made on the same individual.

(*c*) *The changes occurring during the growing period of the locust's life*

In the previous sections the basis for linking measurements together has been that they were made on the same individual. This link is absent here since different individuals are used in successive instars. The trend lines of individuals from successive instars are linked together to form a life curve of weight and oxygen consumption on the basis that their values for 'a' for weight and oxygen consumption fall on a straight line on a log-log plot. This follows the observation by Butler & Innes (1936) and by Zeuthen (1947) that the oxygen consumption is proportional to a power of the body weight. The curve (Fig. 9) is constructed by taking the values of 'b' and the duration of each stadium of these selected individuals and applying them in the correct order to an animal whose weight on hatching is 12 mg. and whose oxygen consumption is 15 mm.^{3}/hr. The curve shows clearly the close correlation between weight and oxygen consumption in the young animal, its absence in the older locust, and its reappearance in adult life in the steady period when the trends for both are horizontal.

DISCUSSION

The discussion will be confined to the relationship between the changes in weight and oxygen consumption which occur in periods of short duration. From the facts given above it is possible to understand the relationships between these two qualities in the intact organism. This can be aided by setting up models of two extremes, the first in which a locust feeds for short though not necessarily regular periods and the second where the meals are separated by long time intervals.

In the first model we start with a locust whose weight and oxygen consumption are at the mid-point of the fluctuations they can undergo and which has a moderate amount of food in its gut. If in this animal the intervals between the meals are short, less than the time taken to empty the crop, which according to Voskresenskaja (1936) is 2 hr., then the midgut can always be kept full by food passing to it from the crop. The digestion and absorption of food from the midgut may be expected to remain constant so that there will be no fluctuation in metabolism on this account. The oxygen consumption will not fluctuate, and when the trend line is removed will appear as a horizontal straight line. Thus there is a method by which the intermittent increase of weight can be smoothed so that its effect on oxygen consumption is eliminated and the changes in the latter can be represented by a smooth curve. This will only be true for absolute values of oxygen consumption, since, if oxygen consumption is expressed as a function of weight, the intermittent increase in weight will affect the oxygen consumption curve. It is assumed here that the animal must feed at intervals of less than 2 hr., but, providing the meals are more frequent than the time taken for the food from one meal to be depleted to the state where oxygen consumption is affected, the actual time interval is probably not important.

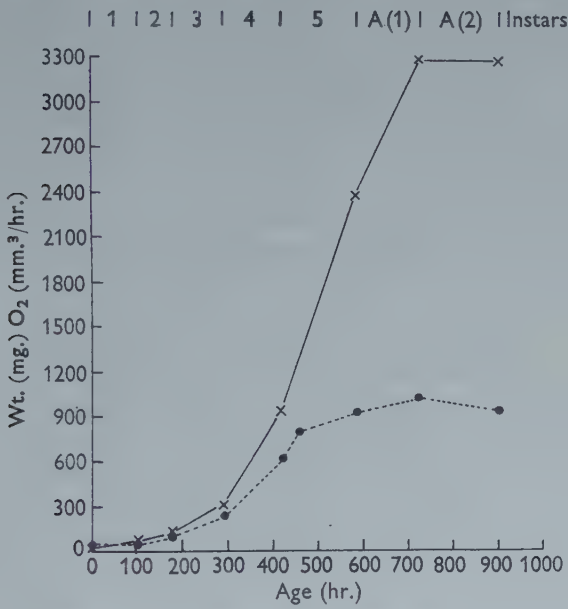

Fig. 9. Changes in weight = × (mg.) and oxygen consumption = ● (mm.³/hr.) throughout the growing period of the locust's life. For further explanation see text. Values for the formula $y = a + b\,(x - x')$, are

Instar	Weight *a*	Weight *b*	Oxygen *a*	Oxygen *b*	*x′*
1	30·45	0·2860	33·58	0·2241	58·35
2	66·41	0·5378	53·20	0·3374	53·45
3	168·27	1·3447	132·50	1·0326	63·36
4	441·5	2·4619	269·5	1·2177	83·30
5	1314·0	6·1977	—	—	109·16
5 Group A	—	—	690·5	4·3814	43·68
5 Group B	—	—	935·6	1·0702	151·58
A(1)	2036·5	5·9886	1134·3	0·8985	156·00
A(2)	2801·6	−0·0655	1070·8	−1·2430	358·45

In the second model an animal is considered whose gut is empty and whose oxygen consumption has sunk to the lowest level for a fasting period. When this animal feeds it will fill up its gut and crop, eating the largest meal which it is capable of holding. According to Voskresenskaja the crop and gut can be filled in less than 1 hr. As has been shown above, this meal will not cause an increase in oxygen consumption for some 4–5 hr., when oxygen consumption will rise sharply. As the food becomes used up the metabolism and oxygen consumption will fall again to their previous low level (unless the animal feeds), to be stimulated again by the arrival of another large meal. Voskresenskaja (1936) gives 9 hr. as the time taken for the locust to empty its gut. So unless the animal feeds at intervals shorter than this time both weight and oxygen consumption can be expected to show their maximum fluctuations.

The first of these models shows that a smooth oxygen consumption curve for a growing animal is theoretically possible and can be obtained providing the animal eats little and often.

SUMMARY

The weight and oxygen consumption of individual locusts, six from each instar, have been measured at 12-hourly intervals throughout each stadium.

An analysis of short duration changes shows that fluctuations in live weight are unavoidable in the growing animal, and that they are accompanied by fluctuations in oxygen consumption. In time the latter lag behind the former.

As the fluctuations of these two variables are out of phase, there is a considerable range of oxygen consumption which must be regarded as normal for any given weight. Calculations are made to find the limits of these fluctuations compatible with normal growth.

The changes that occur during a stadium can be represented by a straight trend line for both weight and oxygen consumption. Exceptions occur in the case of weight of the adult where two straight lines are necessary, one for the growing phase and one for the steady phase of this instar. In certain cases in the fifth instar two straight lines are necessary to express the trend in oxygen consumption which may show an abrupt change in the middle of the stadium.

A high degree of correlation is found between the trend lines for weight and oxygen consumption in the early instars. A low degree is found in latter instars where the weight increases and the oxygen consumption remains nearly constant.

A curve representing the changes of weight and oxygen consumption that occur during the growth of the locust has been constructed.

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THE EFFECT OF HUMIDITY ON THE ACTIVITY OF TSETSE FLIES

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Preliminary to an investigation of the water balance of tsetse flies some experiments were carried out to determine the effect of humidity on general activity. Observations previously made on this aspect of behaviour (Jack, 1939; Mellanby, 1936) have been too cursory to admit of general conclusions.

MATERIAL AND METHODS

Most of the experiments were carried out with *Glossina morsitans* Westwood. The flies were introduced into glass dishes about 20 cm. in diameter and 7 cm. high, one fly to each dish. The dishes were covered with glass lids and fitted with false floors of muslin stretched on wire frames about 2 cm. below the lids. Underneath the false floor were a number of small receptacles for solutions of potassium hydroxide or calcium chloride which controlled the humidity of the chamber.

The chambers were placed at the bottom of a box lined with white paper, twelve chambers being used for each experiment. Continuous with the sides of the box were black curtains hung on a four-sided frame which had at its centre supports for a reflector and lamp. The lamp was about 2 m. from the top of the behaviour chambers, and the intensity of illumination was varied by using stronger or weaker globes. A water filter was interposed below the reflector to absorb heat radiation.

Under these conditions tsetse flies were found to be extremely inactive; quite often a fly would stay in the same place for 10 hr. or more. To get an estimate of activity it was clearly necessary to use extended periods of observation. The procedure finally adopted was to record the position of flies relative to grids marked out on the lid of each dish. Readings were taken at 10 min. intervals every alternate hour from 08.00 to 17.00 hr.; this gave a total of thirty position records for each fly, and from these an empirical measure of activity was derived, namely, the number of records which showed a change in position of flies during the preceding 10 min. expressed as a percentage of the total number of changes which could have been recorded, i.e. thirty. This measure will be referred to as the percentage activity and gives an indication of the frequency with which flies tend to move under different conditions.

χ^2 analysis of the raw data was used to determine the significance of differences in percentage activity, the number of 'active' records and the number of 'inactive' records being set out in 2×2 tables for any pair of conditions.

During the greater part of this investigation temperature could not be controlled and it would rise in the course of an experiment from about 25 to about 30° C. Later on a constant-temperature room became available and a series of experiments was carried out at 25° C. (see Table 2); the results differed in no way from those obtained earlier.

The activity of teneral flies (that is, flies which have not yet had a blood meal) was invariably high during the early morning, falling to a minimum about noon and rising again to high levels at 17.00 hr. when experiments were discontinued. These fluctuations occurred whether the temperature was constant or not, and they may represent some inherent rhythm of activity. But for the purpose of studying the effect of humidity they have been ignored, and values represent the mean activity for the experimental period as a whole.

RESULTS

(1) *The effect of humidity on activity*

Fig. 1 *a* shows the activity of unfed *G. morsitans* at different humidities, and it is clear that the flies are more active in dry than in wet air. In the terminology of Fraenkel & Gunn (1940) the tsetse shows an orthokinetic reaction to humidity, with the eccritic humidity at or near saturation. It must be emphasized that only one part of the orthokinetic complex is dealt with by present methods (Ewer & Bursell, 1950); whether the speed of movement as well as the frequency of movement is affected by humidity has not been determined.

The high level of activity in dry air as compared with wet is maintained throughout the experimental period. In other words, sensory adaptation is not involved in the orthokinetic reaction, or if it is, it occurs too rapidly to be detected by present methods.

The relation between activity and humidity is influenced by the physiological state of the insect. If flies are tested on the day after emergence the increase in activity with decreasing humidity is more or less rectilinear (see Fig. 1 *a*, curve E_1). If they are kept till the second day after emergence the activity is increased at all humidities below saturation; that is, the intensity of the reaction, as measured by the difference between activity in wet and dry air, is increased (Fig. 1 *a*, curve E_2). Three-day-old tenerals tend to be still more active in dry air, but differences could not be statistically established, and the results have not been included in the graph.

Fig. 1 *b* shows the effect of starvation on the humidity reaction of flies which have had one blood meal. On the day after a feed the flies are almost completely inactive, moving on an average once in 15 hr. (see curve F_1). Flies tested 2 days after a feed are somewhat more active, but as yet there is no effect of humidity on activity (F_2). On the third day after feeding the orthokinetic reaction is well established (F_3) and it increases with further starvation (F_4).

Thus both teneral and non-teneral flies show an increase in the intensity of the orthokinetic reaction during starvation. In addition a consistent tendency has been noted towards a change in the form of the activity curves after prolonged starvation.

This is reflected in a disproportionate increase in activity at 80 % R.H. as compared with lower humidities; the result is a hyperbolic rather than a rectilinear relation between humidity and activity (see Fig. 1, curves E_2 and F_4). A change of this kind would have the effect of aggregating the animals more efficiently in regions near the eccritic humidity.

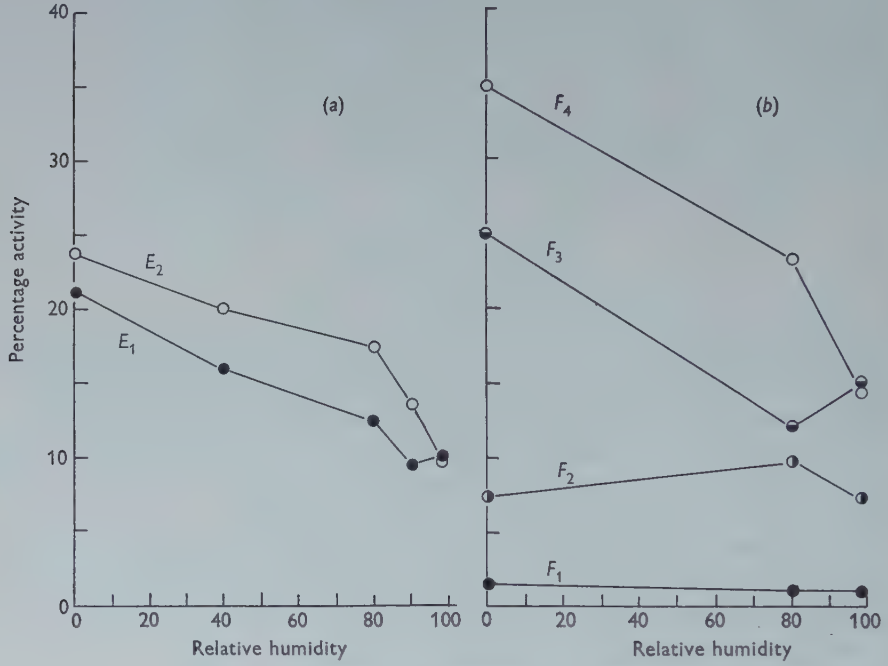

Fig. 1. The effect of humidity on the activity of *G. morsitans*. (*a*), the activity of unfed flies. E_1, 1-day-old flies; E_2, 2-day-old flies. Flies were maintained at 80 % R.H. after emergence; 15–20 different flies were used at each humidity. All differences greater than 4 are significant at the 0·05 level of probability. (*b*), the activity of flies after the first blood meal. F_1, 1 day after feed; F_2, 2 days after feed, etc. Flies were maintained at 50 % R.H. after feeding; ten different flies were used at each humidity. All differences greater than 8 are significant at the 0·05 level of probability.

During starvation in unsaturated air both water content and fat content decrease; to determine which of these variables might be causally related to the changes in the orthokinetic reaction an experiment was carried out with two groups of teneral flies. One group was maintained for 2 days at 50 % R.H., the other at 100 % R.H., and activities were then determined in wet and dry air. The results are given in Table 1 and show that if flies are starved in saturated air there is no significant reaction to humidity, but if they are starved in 50 % R.H. they show a very strong reaction. Analyses of parallel groups of flies show that the two groups do not differ significantly in respect of fat content, but that the water content of the group kept at 50 % R.H. is much the lower. It may be concluded that the changes in reaction intensity which occur during starvation are related to a depletion of water reserves.

Table 1. *The intensity of the orthokinetic reaction of 2-day-old flies after starvation at different humidities*

Starved in	Activity (%)		N	Diff.	P	Water content	Fat content
	0% R.H.	98% R.H.					
50% R.H.	46·3	15·8	8	30·6	0·001	71·00 ± ·84	14·5 ± 1·9
100% R.H.	15·4	12·9	8	2·5	0·5	74·74 ± ·26	12.0 ± 1·7
Diff./S.E.						4·19	1·00

S.E. = standard error of the difference. Water content is given as a percentage of fatless weight; fat content as a percentage of dry weight. In this and subsequent tables N is the number of different flies used at each humidity; since each fly gives thirty position records the total number of records at each humidity in the above table is 240; given this figure, the raw data, that is, the number of 'active' and 'inactive' records, can be calculated for any experiment.

(2) *The effect of light on the orthokinetic reaction*

The experiments described above were carried out with a 300 W. globe as the source of illumination. The same results were obtained with 100 and 75 W. globes, the difference between activities in wet and dry air being 15·0 and 11·7% respectively ($N = 10$ and $P = 0·01$ in both cases); but at still lower light intensities the orthokinetic reaction disappears completely—with a 40 W. globe the difference between activities in wet and dry air was 1·7% ($N = 10$; $P = 0·7$), and at intensities so low that the flies could only just be seen it was 2·5% ($N = 12$; $P = 0·5$). This abolition of the humidity reaction at low light intensities appears to be an all-or-nothing effect, since the intensity of the reaction does not increase and decrease progressively above and below the critical intensity.

An absolute measure of the critical intensity could not be made, but using a Weston Light Meter it was found to be equivalent to the light from a cloudless sky at sunset (i.e. about 12 units).

The effect of low light intensities in abolishing the orthokinetic reaction was confirmed by determining the activity of blinded flies in dry and wet air (see Table 2A); the general level of activity was reduced by the treatment, but there was no difference between activities in wet and dry air. Blinding of the compound eyes alone had the same effect as blinding both compound eyes and ocelli, whilst blinding of the ocelli alone had no effect on the orthokinetic reaction. It is clear that light exerts its effect on the humidity reaction by way of the compound eyes.

Some experiments were done with flies in which the ocelli had been extirpated instead of blinded; the flies appeared to suffer no ill effect from the treatment and showed a strong reaction to humidity thus confirming that the ocelli are not implicated in the light effect. But the general level of activity was almost doubled by ocellectomy. This increase in activity was confirmed at light intensities below the threshold for the humidity reaction (see Table 2*B*). The results seem to be at variance with the concept that the ocelli represent some kind of stimulatory organ (see Wigglesworth, 1953, p. 192).

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The breakdown of the orthokinetic reaction of *G. morsitans* at low light intensities is perhaps related to the markedly diurnal habits of this species (see, for example, Nash, 1933); in insects which display reluctance to move during the night the persistence of a kinetic reaction to humidity after dark would be of little value.

Table 2. *The effect of blinding on the kinetic reaction to humidity*

Treatment	% Activity		Diff.	*P*
	0 % R.H.	98 % R.H.		
A. 100 W.				
1. Complete blinding	17·8	14·4	3·4	0·5
2. Compound eyes blinded	16·1	22·2	+6·1	0·2
3. Ocelli blinded	37·8	24·3	13·5	0·01
4. Ocelli extirpated	51·7	40·5	11·2	0·05
5. Controls	37·2	17·3	19·9	0·01
B. 10 W.				
1. Ocelli blinded	39·4	31·7	7·7	0·1
2. Ocelli extirpated	49·5	46·6	2·9	0·7
3. Controls	31·1	25·5	5·6	0·3

Flies were kept for 1½ days after emergence in 70 % R.H.; they were blinded with a mixture of paraffin wax and Sudan Black and allowed 24 hr. to recover from operative shock. All experiments were carried out in a constant-temperature room at 25° C. $N=6$ throughout.

(3) *The humidity receptors*

An attempt was made to identify the receptors responsible for the orthokinetic reaction, and the results of all experiments are shown in Table 3. The effect of each treatment was determined with reference to a concurrently tested control group.

Table 3. *The effect of various treatments on the intensity of the kinetic reaction to humidity*

Treatment	% activity		*N*	Diff.	*P*
	0 % R.H.	98 % R.H.			
Antennae covered with paraffin wax:					
Experimental	20·0	4·7	12	15·3	<0·01
Control	21·2	11·4	16	9·8	<0·01
Dealation:					
Experimental	22·5	11·9	12	10·6	<0·01
Control	24·2	10·3	12	13·9	<0·01
Tarsi covered with 'Duco':					
Experimental	28·6	19·6	10	9·0	<0·01
Control	23·0	13·6	12	9·4	<0·01
Spiracular filters extirpated:					
Experimental	46·5	47·1	14	−0·6	0·7
Control	29·1	15·0	14	14·1	<0·01

The tests were made with 2-day-old tenerals maintained at 90 % R.H. since emergence; a 24 hr. period was allowed for recovery after treatment.

Covering the antennae with wax caused a decrease in the general level of activity; but there was still a marked orthokinetic reaction, the difference between activity in wet and dry air being, if anything, greater than in the controls. The depressive effect of the treatment may perhaps be ascribed to the damping of a whole afferent field, leading to a decrease in central nervous tone; but it is clear that the receptors concerned with the kinetic reaction to humidity are not a part of this sensory field.

Thin-walled sense organs occur on the leading edges of the wings (Dr C. T. Lewis, personal communication), and on the tarsi (Lewis, 1954); but neither of these types of receptors are responsible for the orthokinetic reaction, since an affect of humidity on activity persists after the wings have been removed and after the tarsi have been lacquered with 'Duco'.

The thoracic spiracles of the tsetse fly are guarded by rows of branched hairs, the 'Reusenhaare' of Geigy & Huber (1952). To extirpate these spiracular filters they were first imbedded in droplets of paraffin wax melted on the loop of an electrically heated wire, and then scraped off with a flattened and sharpened needle. Flies treated in this way showed a marked increase in activity at all humidities, but the higher the humidity the greater the increase, so that there was no difference between activity in wet and dry air. This experiment has been repeated under different conditions of illumination with the same result.

The operation had no apparent effect on the well-being of the flies; they were able to feed normally and lived as long as control flies under given conditions. Nor was the rate of water loss of operated flies greater than that of controls, showing that there has been no interference with spiracular control of water loss (see Bursell, 1956). It seems reasonable to conclude that these spiracular filters are the receptors responsible for the kinetic reaction to humidity.

The spiracular filters have been described and figured by Geigy & Huber (1952); the hairs of thoracic filters consist of stout main stems carrying a number of short side branches, the whole beset with numerous, fine needle-like processes which interlace with those of neighbouring stems. In section the thicker branches are seen to be hollow and they contain, both in old and young flies, a great deal of cellular material. Many of the cells are of typical sensory type with granular cytoplasm, large nucleus and conspicuous nucleolus, and resemble in every respect such sensory cells as occur for instance in association with antennal sense organs. In some cases what appear to be exceedingly fine nerve fibres can be seen to have their origin in these cells. The histology of the thoracic spiracular filter is thus consistent with the view that they are of the nature of sense organs.

The abdominal spiracles also are guarded by filter mechanisms; but here the hairs are of purely cuticular composition, and they are not associated with sensory cells.

The intensity at which the sensory cells are stimulated will be a function of the humidity actually experienced by the 'Reusenhaare'; and since the 'Reusenhaare' are situated at the mouth of the thoracic spiracles this humidity will depend not only on the ambient humidity, but also on the rate at which water is lost through the spiracles. Investigations of spiracular control under conditions of sustained activity have shown that the lower the water content of flies the greater the degree of closure of spiracles (Bursell, 1956); and thence, at a given humidity, the drier the conditions to which the humidity receptors are exposed. The observed increase in the frequency of movement during starvation in unsaturated air (see Fig. 1) is perhaps a reflexion of this phenomenon, assuming that spiracular control occurs during rest as well as during activity. Furthermore, the difference in degree of spiracular closure between 1 and 2-day-old flies is greater at about 80 % R.H. than at lower humidities; this is in accord with the observation that at this humidity occurs the greatest increase in activity. It seems that the changes in intensity and form of the orthokinetic reaction described in § (1) may be a simple consequence of the association of humidity receptors with the thoracic spiracles.

The results of extirpation experiments show that impulses from the humidity receptors cause central inhibition of locomotion; for when these impulses are cut off by destroying the sense organs, the level of activity is increased. An analogous situation seems to hold for *Ptinus tectus* in which elimination of the humidity receptors causes an increase in the activity of the insect at the eccritic humidity (Bentley, 1949). In *Tenebrio*, on the other hand, the orthokinetic mechanism appears to be based on excitation in so far as the removal of humidity receptors causes a decrease in activity in wet air (the insect has a dry preference) (Pielou, 1946), and the same applies to the reaction away from saturation in *Peripatopsis* (Bursell & Ewer, 1950).

(4) *The orthokinetic reaction in different species*

The effect of humidity on activity has been studied in five species of tsetse; the results for 2-day-old flies are shown in Table 4. In all the activity is greater in dry than in wet air; *G. pallidipes* Austen has a significantly stronger reaction than the other members of the *morsitans* group. The reaction intensity of *G. palpalis fuscipes* (Robineau-Desvoidy) is intermediate, but the general level of activity is twice as high as in the other species. Since this difference cannot be demonstrated in the dark, it is thought to represent an intolerance on the part of a forest fly to the high light intensities used in the experiments.

Table 4. *The orthokinetic reaction in different species of tsetse fly, two days after emergence*

Species	% activity		Difference	*N*
	0 % R.H.	98 % R.H.		
I. *Morsitans* group:				
G. morsitans morsitans	23	9	14	20
G. swynnertoni	24	14	10	16
G. austeni	33	18	15	8
G. pallidipes	48	20	28	12
II. *Palpalis* group				
G. palpalis fuscipes	79	61	18	11

All differences are significant at the 0·001 level of probability; and *G.p. fuscipes* is significantly different from all the members of the *morsitans* group at both humidities. The flies were maintained at 80 % R.H. after emergence.

(5) *Difference between the sexes*

Table 5 shows that in unfed flies there is no significant difference of activity between male and female flies; but in fed flies the males are more active than the females. These results are in accord with field experience; for instance, Jackson (1946), working with an isolated generation of *G. morsitans*, showed that whilst there is little difference in the activity of male and female before the first blood meal, in later life the females are relatively inactive. They are contrary to the findings of Mellanby (1936), that newly emerged females are much more active than newly emerged males of *G. palpalis*.

Table 5. *The percentage activity of males and females*

Species	% Activity				*P*
	Males	*N*	Females	*N*	
G. swynnertoni unfed	18·4	33	20·6	15	0·5–0·3
G. morsitans unfed	16·1	48	14·9	45	0·5–0·3
G. morsitans fed	19·8	48	16·0	54	0·01

The values represent the sum of activities at three different humidities.

DISCUSSION

There is still some doubt about the natural habits of the East African tsetse flies. Most field studies are based on the behaviour of the non-teneral male in its relation to a catching party. Where it perches and how it behaves in the absence of such catching parties are still open questions. This paucity of information makes it difficult to relate laboratory studies to the life of flies in the field. But the problem may be approached from another angle, by asking what are the environmental conditions obtaining in the field to which any response of the fly must be related. Taking the savannah flies as an example, the answer as far as humidity is concerned seems to be that differences between different parts of the habitat are very slight, probably of the order of 10% R.H., if that (see, for example, Buxton & Lewis, 1934, Fig. 13). The humidity of special sites, such as rot holes, may differ more from that of the general environment, but flies are very rarely encountered in such sites (Burtt, 1955). Thus there is an apparent discrepancy between the occurrence of a well-marked orthokinetic reaction to humidity on the one hand, and on the other the lack of opportunity under natural conditions of encountering differences in humidity sufficiently great to make such a reaction of significance.

This anomaly could be resolved by considering the possible effect of the fly on the microclimate of its perching place. The normal rate of water loss of a resting tsetse fly is about 0·16 mg./hr. (unpublished observation), and, since 1 c.c. of saturated air at 25° C. contains about 0·02 mg. of water, in 10 min. enough water would be lost to saturate 1·3 c.c. of air. A fly selects a perching place in response perhaps to visual and tactile stimuli; if that perching place is exposed so that the water lost from the fly is rapidly dissipated, the air surrounding the fly will remain dry and the orthokinetic reaction will come into play to ensure that the insect moves quickly to another site. If, on the other hand, the fly perches in a well-protected crevice in the bark of a tree (Swynnerton, 1921; Lamborn, 1916), the water vapour lost may be to some extent retained in the vicinity of the fly, the ambient atmosphere will become moist and activity will be inhibited. In other words, there seems a possibility that the tsetse fly may produce its own microclimate as far as humidity is concerned, and that the significance of the orthokinetic reaction is to be sought in these terms.

An investigation is in progress of the transpiration of resting tsetse flies in still and moving air, and it is hoped that results will throw further light on this possibility. In addition an attack is being made by this Organisation on the vexed question of natural perching places, and the extent to which sheltered places are frequented by flies in the field will be investigated.

SUMMARY

1. The activity of hungry tsetse flies is greater in dry air than in wet, and the intensity of this orthokinetic reaction increases in the course of desiccation.

2. The orthokinesis is abolished at low light intensities; this effect of light is mediated by the compound eyes.

3. The thoracic spiracular filters are the sense organs responsible for the kinetic reaction to humidity.

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EFFECT OF PHOSPHATE AND CHLORIDE IONS ON AEROBIC METABOLISM OF BOVINE SPERMATOZOA

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INTRODUCTION

The oxygen consumption of mammalian spermatozoa is influenced by the type of diluent used. Both the respiratory rate and the motility of spermatozoa are greater when the semen is diluted with isotonic sodium chloride than with phosphate buffer. Although the pH of the unbuffered saline falls to pH 5·62, no deleterious effects are observed.

The present report extends the observations of Bishop & Salisbury (1955*b*). The data indicate that the aerobic metabolic activity of bull spermatozoa varies in isosmotic solutions of sodium salts according to the presence of chloride or phosphate ions.

MATERIALS AND METHODS

The collection of semen, estimation of cell concentration and semen pH have been previously described (Bishop & Salisbury, 1955*a*). A total of sixty-eight individual semen samples was collected, of which fifty three were used in the direct comparison of two diluents. Twenty-four of these semen samples were collected from 17 May to 21 July 1954, six from 16 October to 4 November 1954, and twenty-three samples from 21 January to 16 February 1955. The diluents used were isotonic (0·154M) sodium chloride and an isosmotic phosphate buffer at pH 7·0 (701·3 ml. of 0·13M-$Na_2HPO_4 . 12H_2O$ and 280·0 ml. of 0·17M-$NaH_2PO_4 . H_2O$). The osmotic pressure of both diluents, determined by freezing-point estimations in a Fiske osmometer, was 288 ± 5 milliosmols.

In most tests the metabolism of the spermatozoa was measured in diluted whole semen, but in some cases the cells were washed with isotonic sodium chloride or phosphate buffer. Whole semen was centrifuged for 10 min. at 1500 r.p.m. and the supernatant seminal plasma removed. Sodium chloride solution or phosphate buffer was added to restore the original volume and the spermatozoa were gently resuspended by drawing up and down in a large bore Pasteur pipette. The resuspended cells were again centrifuged, the supernant fluid was removed and replaced with fresh diluent. The process was repeated once more to obtain twice-washed cells suspended in a medium free from seminal plasma. In the subsequent dilution of the cells isotonic sodium chloride was added to those cells washed with sodium chloride and phosphate buffer to phosphate-washed cells.

Oxygen uptake was measured in air at 37° C., over a 4 hr. period by the direct Warburg method (Umbreit, Burris & Stauffer, 1949); 20% KOH was used in the centre well to absorb CO_2. The shaking rate was 110 oscillations per minute.

One part of semen or washed cells was diluted with 4 parts of diluent in the Warburg flasks. In order to measure immediate or transitory effects on respiration, 0·8 ml. of diluent was placed in the flask, and after a 10 min. equilibration period 0·2 ml. of semen or cells was added from the side arm.

Aliquots were taken at the beginning and end of each experiment to determine the fructose and lactic acid content of the suspensions. Fructose was determined by the method of Roe (1934) as modified by Mann (1948), and lactic acid by the procedure of Barker & Summerson (1941).

In an attempt to demonstrate the hexosemonophosphate pathway, a cell-free extract of sperm cells was prepared by disrupting the washed cells suspended in saline, in a 10 kV. Raytheon sonic oscillator at 9000 c./s. for 10 min. with glass beads. Glucose-6-phosphate (G-6-P) dehydrogenase or 'Zwischenferment', prepared by the method of Kornberg (1950), served as the standard of comparison. The optical density change of triphosphopyridine nucleotide (TPN) reduction was observed at 340 mμ with the Beckman Model DU Spectrophotometer.

RESULTS

(1) *Effect of phosphate and isotonic sodium chloride diluents on semen*

The effect of sodium chloride and phosphate diluents on the average rate of O_2 uptake, fructose utilization and the accumulation of lactic acid is summarized in Table 1.

Table 1. *Effect of diluent on the metabolism of bovine semen*

Diluent	Final motility		Aerobic activity			$\frac{Z_{\text{Lactic}}}{Z_{\text{Fruct.}}}$ (%)
	%	Rate	$-Z^{*}_{O_2}$ (μl.)	$-Z_{\text{Fruct.}}$ (μg.)	$+Z_{\text{Lactic}}$ (μg.)	
Sodium phosphate	26 ± 15	1 ± 1	5 ± 2	42 ± 27	27 ± 22	65
Sodium chloride	44 ± 13	2 ± 1	10 ± 3	35 ± 26	9 ± 21	26

* Z = activity/10^8 spermatozoa/hr. Redenz (1933).

Mean of fifty-three semen samples from twelve bulls. Initial motility 51 ± 9 %. Initial rate of motility 3 ± 0. Mean cell count $2·77 \times 10^8 \pm 0·73$ cells/ml. of diluted semen.

The average fructose utilization during aerobic incubation was about 20 % higher in the phosphate than in the chloride diluent, and more lactic acid was accumulated in the phosphate diluent. The increase of lactic acid determined at the end of the incubation period averaged 65 % of the fructose utilized in the phosphate and 26 % in the saline diluent. However, the variability observed, as indicated by the standard deviations shown in Table 1, was large. One hundred per cent or more of the fructose utilized was found as lactic acid in fifteen of the fifty-three semen samples diluted in phosphate and in only nine diluted in saline.

When these semen samples were grouped according to periods of collection the following differences were noted. The average lactic acid determined for the fructose utilized was 59·1 % for twenty-four phosphate-diluted semen samples collected in May, June and July. On the other hand, the accumulation of lactic acid averaged 4·3% in chloride-diluted samples. In the October–November collections the average accumulation was 100% for the six semen samples diluted in each diluent. For the January–February collections, the average was 62% in the phosphate and 33% in the chloride solution. The reasons for these differences in the metabolic behaviour of sperm cells with the seasons of the year are not known.

The fifty-three semen samples collected from twelve bulls were not distributed equally in the different seasons of the year. Thus, a random selection of the data was made to obtain an unbiased estimate of the effect of the diluents and season.

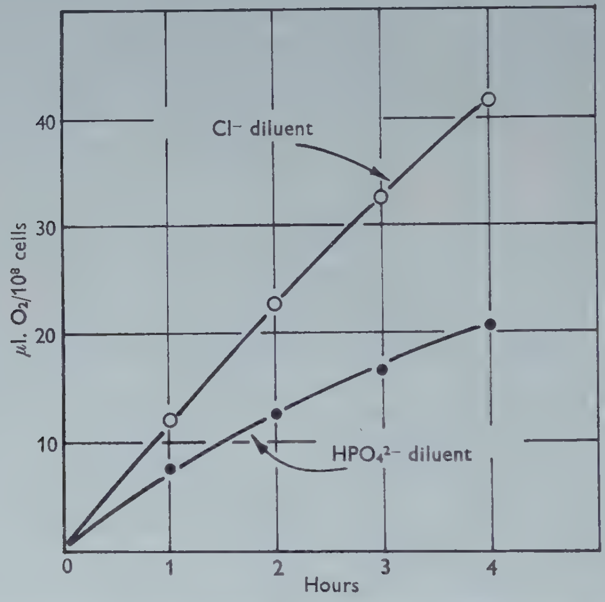

Fig. 1. Effect of diluent on semen respiration. Mean oxygen uptake of fifty-three semen samples diluted 1:4 with 0·9% saline and isosmotic sodium phosphate at pH 7·0.

Thirty samples were proportionally distributed among the bulls for the analysis of variance of these data. It was found that the time of the year did not affect the oxygen uptake but that the differences in fructose utilization and lactic acid formation from one season to another were significant ($P<0{\cdot}01$ and $<0{\cdot}05$ respectively). Between the two diluents, chloride and phosphate, the differences of O_2 uptake and lactic acid formation were both highly significant ($P<0{\cdot}01$), but for fructose utilization no significant difference ($P>0{\cdot}05$) was present.

Characteristically, the Z_{O_2} values in the phosphate diluent averaged about one-half as much as those in the chloride diluent. The initial rate of oxygen consumption (Fig. 1) was maintained without appreciable change throughout the 4 hr. period in the chloride diluent; while that in the phosphate diluent was less and declined somewhat more rapidly. Motility of the cells was also maintained better in samples diluted with chloride than in those diluted with phosphate.

(2) *The effect of washing spermatozoa with sodium phosphate or isotonic sodium chloride and subsequent dilution*

During the January–February collection period, when the total volume of the individual ejaculates permitted, one-third of each sample was used for the experiments reported above. The other two-thirds were washed twice, one-half with phosphate diluent and the other half with chloride diluent. Each of these was subsequently resuspended in the respective diluent to give approximately the original cell concentration. These were then split into two parts and diluted 1:4, one with the diluent used for washing plus 2·0 mg. of fructose per ml., and the other with seminal plasma. The seminal plasma, which had been withdrawn after the first centrifugation, was diluted in the proportion of 0·2 ml. plasma to 0·6 ml. of the diluent and added to the cells.

The process of washing reduced the percentage of motile cells by about 5% in chloride diluent and slightly more in the phosphate. The results of the metabolic measurements, the final percentage of motile cells, and the rate of motility after 4 hr. are given in Table 2.

Table 2. *Effect of washing and dilution on the metabolism of bovine semen*

Treatment	Diluent	Final Motility		Aerobic activity			$\frac{Z_{\text{Lactic}}}{Z_{\text{Fruct.}}}$ (%)
		%	Rate	$-Z_{O_2}$ (µl.)	$-Z_{\text{Fruct.}}$ (µg.)	$+Z_{\text{Lactic}}$ (µg.)	
None	HPO_4^{2-}	22	1	5·0	50·2	31·4	62·5
None	Cl^-	41	2	9·3	44·1	14·5	32·9
HPO_4^{2-} washed	HPO_4^{2-} + fruct.	8	0	4·5	12·3	17·1	139·0
HPO_4^{2-} washed	HPO_4^{2-} + S.P.*	25	1	6·2	30·2	57·3	189·7
Cl^- washed	Cl^- + fruct.	30	2	10·8	26·6	4·3	16·2
Cl^- washed	Cl^- + S.P.*	39	2	9·2	36·9	26·3	71·3

* S.P. = seminal plasma. Mean of twenty-three semen samples.
Initial motility: unwashed, 51% + 3; Cl^- washed, 46% + 3; HPO_4^{2-} washed, 44% + 3.
Mean cell count $2·55 \times 10^8$ cells/ml. of diluted semen.

The respiration rate and fructose utilization were lower in samples washed in phosphate, diluted in phosphate-fructose (or in seminal plasma plus phosphate) than in those in which the phosphate was replaced with chloride. However, the proportion of lactic acid level in relation to the fructose utilized was greater than could be accounted for by the loss of fructose. When carbohydrate was added to washed cells suspended in chloride, about one-sixth of the fructose utilized was detected as lactic acid. But in the presence of seminal plasma a much higher proportion of lactic acid accumulated.

(3) *Effect of varying osmotic pressures with phosphate and chloride diluents*

The oxygen consumption data for these experiments, conducted with semen collected in May and June and diluted 1:4 with either phosphate or chloride solutions of varying concentrations, are shown in Fig. 2. Eleven ejaculates provided the data for each point of curve *A*. Four other individual semen samples, two of which were of sufficient volume for complete duplications, provided the data for curve *B* which illustrate the repressive effect of phosphate concentration on respiration.

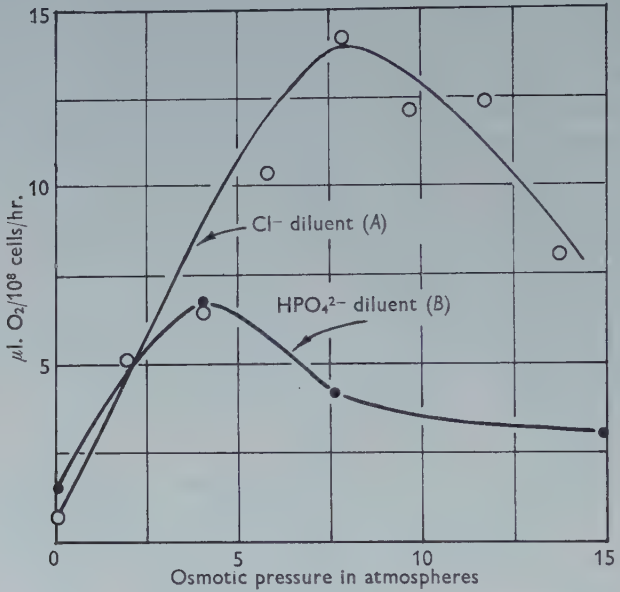

Fig. 2. Effect of osmotic pressure on semen respiration.

The greatest Z_{O_2} was at a concentration of chloride having an osmotic pressure (approximately 8 atmospheres) similar to that of semen. [The osmotic pressure of semen is 7·2–7·6 atmospheres at 37° C. (Salisbury, Knodt & Bratton 1948; Rothschild & Barnes, 1954).] Fructose utilization and lactic acid accumulation were also greatest at that osmotic pressure. In curve *B* the O_2 uptake parallels that of curve *A* up to a molar concentration of 0·05 M (approximately 3·9 atmospheres); then an inhibitory effect supervenes. The fructose utilization and lactic acid accumulation were again greatest not at the point of greatest O_2 uptake but at the approximate osmotic pressure of semen.

(4) *Addition of catalase to whole semen*

In order to eliminate the possible oxidation of amino-acids as a source of H_2O_2 (Tosic & Walton, 1950), which depresses motility and oxygen consumption in the phosphate solution, a series of samples was split four ways with two portions of each suspended in chloride diluent and two in phosphate diluent. To one of the sub-samples of each diluent crystalline catalase was added to a final concentration of 1:10,000. The added catalase depressed respiration slightly in the phosphate (by an average of 9 %) and increased it slightly in the chloride (by an average of 5 %). The essential differences between the two diluents in the metabolism of semen, however, were not altered by the addition of catalase. Thus the formation of toxic amounts of H_2O_2 from oxidation of amino-acids or of other substrates does not seem to be the primary cause of the inhibition of respiration and motility observed in the phosphate diluent.

(5) *Tests for the hexosemonophosphate pathway*

Experiments were undertaken to demonstrate the presence of the hexosemonophosphate pathway. The oxidation of glucose-6-phosphate (G-6-P) with triphosphopyridine nucleotide (TPN) as the hydrogen acceptor yields 6-phosphogluconate (6-PG) and reduced triphosphopyridine nucleotide (TPN.H).

Observations indicated that TPN reduction either did not occur—or amounted to less than 0·005 μM/10 min.—with the sonic extract of twice-washed spermatozoa ($29{\cdot}2 \times 10^8$ cells/ml.). Diphosphopyridine nucleotide (DPN) also failed to serve as a hydrogen acceptor under similar conditions. TPN.H generated by yeast G-6-P dehydrogenase in the presence of a limiting amount of G-6-P was reoxidized in an aerated cuvette at a rate less than 0·004 μM/10 min., indicating that TPN.H dehydrogenase activity was not masking the G-6-P dehydrogenase of the sperm cells. In addition, the rate of G-6-P oxidation by yeast dehydrogenase was the same in the presence or absence of sperm extract. Thus an inhibitor of this reaction was not responsible for the lack of dehydrogenase activity.

The oxygen consumption of the whole semen in chloride diluent, before sonic oscillation, was 8·7 μl./hr./10^8 cells at 37° C. measured in air. On the assumptions that (1) all of the oxidation was mediated through the hexosemonophosphate pathway, and (2) all of the dehydrogenase activity was present in the extract, the theoretical rate of reduction should have been 0·112 μM/10 min. It was concluded that G-6-P dehydrogenase, if present in the cells in such concentration, could have been easily detected in the sonic extracts. However, the reaction was at best 26·8% of the theoretical value.

DISCUSSION

In most cases with the phosphate-diluted semen lactic acid appeared to have been oxidized or not to have been formed since the accumulated lactic acid was less than the fructose utilized. In most of the washed cells diluted in phosphate-fructose diluent the accumulation of lactic acid was greater than the loss of fructose. This would suggest that an endogenous substrate was utilized for the formation of the excess lactid acid.

The lactic acid accumulation of semen and of washed cells, both in chloride diluent, was, however, much lower than when phosphate was used as the diluent. The oxygen consumption in chloride diluent was approximately twice as great as in phosphate, but was insufficient to account for the loss of lactic acid on a quantitative basis, except in one case. Since the addition of catalase to whole semen diluted in chloride or phosphate did not essentially alter the metabolic response, it appeared that the depression of respiration of whole semen diluted in phosphate was not due to H_2O_2. The motility of washed spermatozoa was maintained best in cells washed and resuspended in chloride diluent plus seminal plasma.

Further, a higher percentage of lactic acid accumulated in plasma-containing diluents than in diluted whole semen.

Oxygen may be partly involved in the initiation and maintenance of motility of bovine spermatozoa as in sea-urchin spermatozoa (Rothschild, 1948). Thus in the process of washing, inhibitory substances or waste products are removed and oxygen is more easily available to the organism. However, seminal plasma may contain substances which inhibit oxidative processes; hence the larger accumulation of lactic acid.

Phosphate ions in levels greater than 0·05 M (3·9 atmospheres) have an inhibitory effect on respiration; however, fructolysis appears to be influenced by the tonicity of the diluent. On the other hand, the tonicity of the chloride diluent influenced both respiration and fructolysis. Sperm cells washed and resuspended in chloride diluent utilized more fructose, but accumulated less lactic acid than those in phosphate diluent. Chloride ions apparently favoured the oxidation of lactic acid (or inhibited the formation of lactic acid), whereas phosphate ions, in the concentration used, inhibited the oxidation of this substance.

Though G-6-P and 6-PG dehydrogenase were not demonstrated, Mann (1951) and van Tienhoven, Salisbury, VanDemark & Hansen (1952) reported that intact spermatozoa utilized glucose in the presence of fructose. This would suggest that the hexosemonophosphate pathway may be operative in spermatozoa.

Significant differences were observed in the metabolic pattern of bovine spermatozoa collected during the different seasons of the year. Work is in progress at present to confirm these observations.

SUMMARY

Under the aerobic conditions of these experiments, dilution of bovine semen with phosphate ion depressed oxygen consumption and motility of sperm cells. However, fructose utilization and lactic acid accumulation were increased in the presence of phosphate ion. When semen was diluted with isotonic sodium chloride oxygen uptake was greater, motility was maintained better and fructose utilization and lactic acid accumulation were lower than in the phosphate-diluted semen.

Twice-washed cells in a diluent containing seminal plasma utilized more fructose and accumulated more lactic acid than those in plasma-free diluent. The depressive effect of phosphate on respiration was not due to H_2O_2 formation nor to changes of osmotic pressure.

The season of the year in which semen was collected influenced its metabolic response in each of the diluents studied. The presence of glucose-6-phosphate dehydrogenase activity was not conclusively demonstrated in cell-free extracts of bovine spermatozoa prepared by sonic oscillation.

The authors wish to express their appreciation to Micah Krichevsky and Professor W. A. Wood of this department for their assistance in the work concerning the hexosemonophosphate pathway; and to Professor W. O. Nelson for his suggestions and review of the manuscript.

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AN INTERFEROMETRIC METHOD OF QUANTITATIVE ANALYSIS OF ANTIGEN-ANTIBODY REACTIONS IN GELS

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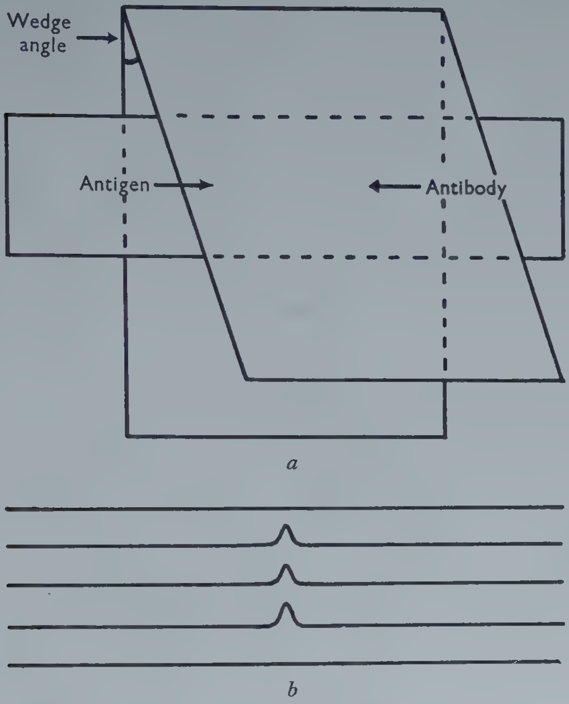
(With Plates 1 and 2)

INTRODUCTION

The most sensitive technique available for analysing complex mixtures of proteins consists of using these substances, by injection into a suitable animal, as antigens, and allowing them to diffuse through a gel containing the antibodies produced by the animal. Each antigen and its specific antibody combine in certain proportions, and if they are present in sufficient amounts a band of precipitate is formed.

Oudin (1946) allowed one reactant to diffuse into a gel in which the other was uniformly distributed. Ouchterlony (1949) and Elek (1948) allowed both components to diffuse towards one another through a gel. Complex mixtures of antigens and antibodies should be capable of analysis by means of these techniques, since each specific antigen-antibody system behaves independently as if the others were not present.

The principles of the interferometric technique have been described by Ambrose (1948), and a similar technique has been used by Robinson (1950) to examine the diffusion of solvents through polymers. It consists essentially of observing the diffusion of the reactants through a column of gel by means of an interference system of the Fabry-Pérot type derived from the multiple reflexions of a beam of light between two reflecting surfaces inclined to form a wedge (Text-fig. 1*a*). Interference fringes are observed, each fringe being a contour line of constant optical path. As diffusion of antigen or antibody proceeds a change of refractive index occurs and the initially straight fringes bend, each fringe representing exactly the refractive index gradient of the diffusing substance. If there is an accumulation of a complex between an antigen and its specific antibody in a particular region, an inflexion in the fringes occurs (Text-fig. 1*b*) in which the peak corresponds to the point of equivalence of the antigen and antibody. The quantity of antigen and antibody localized before commencement of precipitation may be measured directly from the area under the peak and the thickness of the gel. The technique is applicable to extremely complex mixtures of antigens and antibodies, and is also a very sensitive method of qualitative analysis.

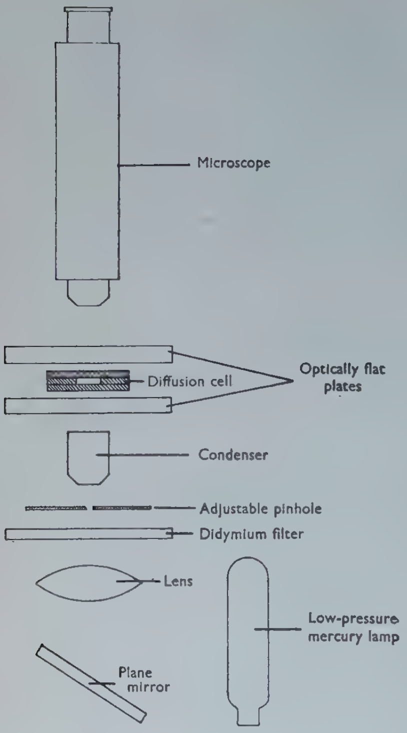


Text-fig. 1. (*a*) Illustrating the principle of the method; and (*b*) indicating the interference fringes in the same orientation.

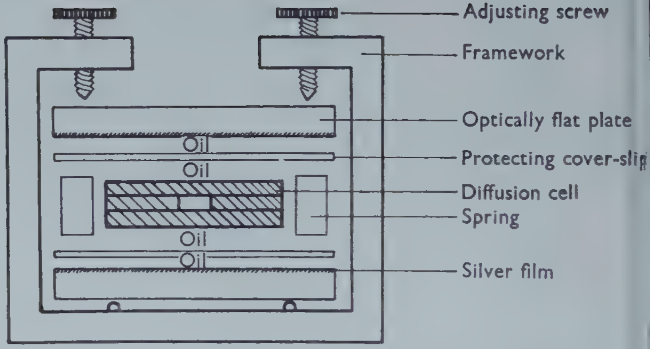
METHODS

The interference system consists of two $\frac{1}{4}$ in. thick glass plates (2 by $1\frac{1}{2}$ in.). Plates of this thickness are used in order to prevent distortion of the fringes when the plates are subjected to the slight pressure which it is necessary to apply to adjust the angle between them. Each plate is optically flat on one surface which is silvered *in vacuo* to give about 10% transmission of white light. The density of the silvering is important since if the film is too dense, insufficient light is transmitted, and if the film is insufficiently dense the lack of contrast in the interference fringes makes observation difficult. The silver film is very easily damaged and deteriorates if exposed to the atmosphere for too long. To overcome these difficulties the silvered surfaces can be protected with large cover-slips cut to size and held on the silvered surface by a thin film of immersion oil. The protection can be made even more secure by sealing the edges of the cover-slip and plates with a cold-setting resin such as 'Araldite'. Protected this way and used carefully, one pair of silvered optical flats will last almost indefinitely. Thin cover-slips were used to protect the silver film rather than thicker glass plates, since the contrast of the fringes decreases as the distance between the silvered surfaces increases, although the sensitivity is increased at the same time.

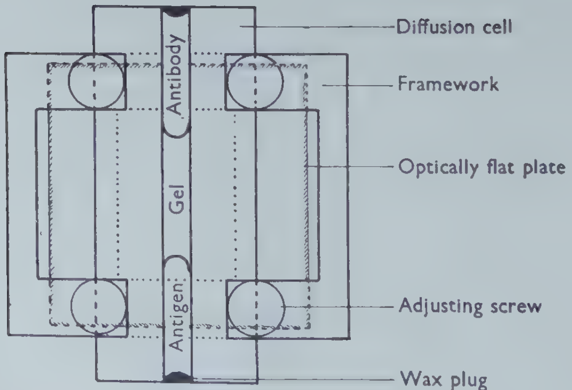
The apparatus is shown diagrammatically in Text-figs. 2–4.



Text-fig. 2. The optical system.



Text-fig. 3. Exploded side view through apparatus.



Text-fig. 4. Top view of apparatus with upper optical plate removed.

The two plates with their silvered surfaces facing one another are placed horizontally on a microscope stage separated by two narrow strips of sponge rubber or thin steel springs leaving a gap of 2–3 mm. The plates are mounted in a rigid brass framework which has four vertical screws which may be screwed down on to the four corners of the uppermost plate, thus permitting the delicate adjustment of the angle between the plates on which the spacing of the fringes is dependent. The object to be viewed is placed between the plates with a film of immersion oil between the object and the plates. The plates are adjusted until they are almost parallel. They are illuminated from below by a beam of parallel monochromatic light from a low-pressure mercury vapour lamp. For photography it was found advantageous to insert a Kodak Didymium filter. The angle of incidence of the monochromatic light, on which the sharpness of the fringes is very dependent, must be adjustable by means of a horizontally movable pin-hole. Very sharp Fabry-Pérot-type fringes are obtained on a practically dark field, each fringe being a contour line of constant optical path between the silvered surfaces of the plates. The wedge angle between the plates is usually adjusted to be at right angles to the direction of diffusion, and as diffusion proceeds there is a displacement of the fringes to maintain the optical path constant.

The diffusions were generally carried out in cells constructed from three microscope slides. One slide was cut along its length into two halves which were placed between two whole slides to form a flat tube about 4 mm. wide, 1 mm. deep and 7 mm. long, open at both ends. For ordinary purposes the best seal was obtained by dipping the edges of the cell, held firmly in a bulldog-clip, into a dish of molten paraffin wax containing an equal quantity of vaseline. It was found that a strong watertight seal was obtained without any wax penetrating into the diffusion channel. If microscope slides having obvious optical flaws were rejected it was found that the slides were sufficiently flat over the small areas utilized to give no distortion of the interference fringes. The molten gel was then injected into the centre of the channel using a hypodermic syringe, care being taken to avoid the formation of bubbles which is not difficult if the channel is free of grease. When the gel had set the antigen solution was injected into the space at one end of the column of gel and the antibody solution at the opposite end. The ends of the channel were then sealed with a little of the molten paraffin wax/vaseline mixture.

The use of gelatin presents no difficulties as it adheres strongly to glass, but agar contracts on setting and often a leakage occurred along one or more of the agar/glass interfaces. This can be overcome in the ordinary gel-diffusion technique by drying a film of agar gel on to the glass before adding the agar diffusion gel. When this was tried it was found that the resulting column of gel, although homogeneous to the naked eye, was far from being so under the interferometer. One way of overcoming this leakage difficulty using agar gel was to allow the agar to set and then to inject gelatin solution into the spaces at either end of the agar column. The excess gelatin solution was carefully drained off using filter paper, and the remainder which wets all the surfaces was allowed to set, thus sealing all the potential leaking interfaces. The thin film of gelatin was found not to retard diffusion to any detectable extent.

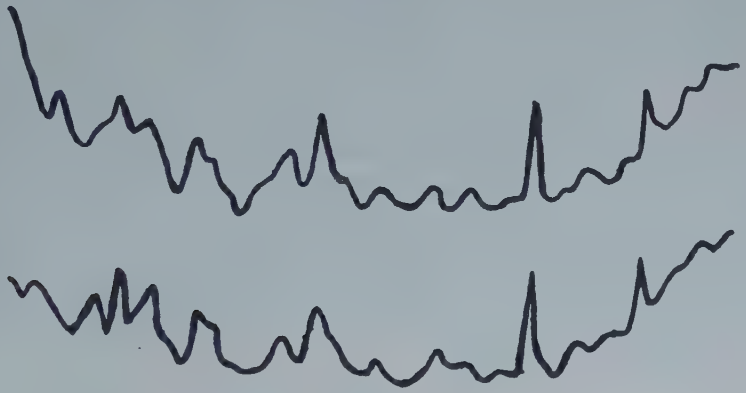
RESULTS

Two main methods have been used by previous workers to characterize antigens present in a mixture. The first, developed by Oudin (1947, 1948*a*, *b*, 1949), consists of allowing the antigenic mixture to diffuse into antiserum which has been set in agar, when a number of precipitation fronts are developed. The distance travelled by these fronts depends on the concentration of the antigenic components, on their diffusion coefficients and on the square root of the time. The diffusion coefficients can be determined from the diffusion test and thus the quantities of antigens may be determined. Becker (1953) and other workers, however, claim that the presence of non-reacting substances can lead to serious errors in the estimation, mainly due to the effect they have on the migration rates of the bands.

The second method, developed by Oakley & Fulthorpe (1953), uses a double diffusion technique. Antiserum is set in agar in the bottom of a tube; on this is placed a central column of agar on which the antigenic mixture is poured. Disks of precipitate are formed in the agar column. If an antigen is diluted its disk appears higher in the column and is fainter. The amounts of antigen present in the mixture may be roughly estimated by finding the smallest amount of it which, when allowed to diffuse through constant antiserum, gives a line in the upper meniscus of the central agar column. Presumably the system is first calibrated by using a solution containing a known concentration of the antigen. Furthermore, the antigens may be characterized by the values of antisera against them, determined by finding the amounts of antisera it is necessary to add to the antigenic mixture to move each line in turn into the upper meniscus, or to cause it to fade out. With mixtures giving a few lines, Oakley has achieved considerable accuracy with this technique, but the more complex the mixture the more difficult it is to apply.

QUALITATIVE APPLICATIONS

Qualitatively, the interferometric method is superior to the techniques at present used to detect the number of bands or fronts formed, particularly when the accumulation of antigen-antibody complex has been too small to result in the formation of a precipitate. This occurs most frequently with 'flocculating' systems but also in 'precipitating' systems. Such regions can often be picked out as Schlieren lines without the aid of the interferometer but if there are several of them close together it is generally impossible to count the number. Pl. 1, fig. 3, shows such a system, which when viewed suitably with the naked eye or ordinary microscope showed a broad region obviously containing a large number of lines which contained no detectable precipitate. It was impossible, however, to form an accurate estimate of the number present. Viewing through the interferometer immediately revealed a large number of small but definite peaks in the fringes, which cannot be caused by an irregular inhomogeneity in the gel as the peaks in the fringes are superimposable, showing that the inhomogeneity runs right across the width of the channel at right angles to the direction of diffusion. This can be seen more clearly in the tracing of the contours of the fringes shown in Text-fig. 5. The multiplicity of the main opaque band on the right of Pl. 1, fig. 3, can be seen in the interferometer but not with the ordinary microscope. Occasionally, very faint diffuse bands were encountered in 'precipitating' systems which were just detectable to the naked eye but did not appear as peaks in the fringes, presumably because they were not contributing sufficiently to the refractive index. Such bands are easily detectable, however, by adjusting the angle of the incident beam so that the fringes almost vanish.



Text-fig. 5. Tracing of the contours of the fringes shown in Pl. 1, fig. 3.

The increased sensitivity of the interferometer over other methods of examination is demonstrated by the observation that the main band of the diphtheria toxin-antitoxin system could be resolved into at least six razor-sharp interference peaks within the narrow band of precipitate, which appears to be single and homogeneous under the ordinary microscope. This is in agreement with the observation of Pope (1954) that crystalline diphtheria toxin contained at least six antigens, although it gave a single line in the diffusion test. Pl. 1, fig. 4, considerably enlarged and taken with the fringes almost completely spread out, shows the complexity of this main band.

QUANTITATIVE APPLICATIONS

The quantitative applications of the method are all based on the measurement of the areas under the peaks formed in the interference fringes by the accumulation of antigen-antibody complex. Fortunately, the refractive indices and specific refractive increments are almost constant for all proteins (Adair & Robinson, 1930), hence the relative and absolute amounts of protein present in each peak in the diffusion cell may be measured. This will be roughly true even for non-protein antigens since the polyvalent antigens generally combine with considerable excess of antibody to form the complex at equivalence point. The measurement of the areas under the peaks is greatly simplified by the use of the camera lucida. Pronounced peaks are easily observed with 'horse' (flocculating) systems (see Pl. 1, fig. 1) since no precipitate is formed until the ratio of antigen to antibody rises to a certain minimal level. Such systems are horse antisera against certain proteins such as diphtheria toxin, serum albumins, ovalbumin and conalbumin. With the 'rabbit' (precipitating) systems the peaks are not so large as precipitate is formed however high the ratio of antigen to antibody may be; and precipitated complex appears as a more or less opaque region in the interferometer. With both systems, once the precipitate has been formed it does not contribute to the refractive index in that region to the extent which it would in the absence of precipitate formation. There are two procedures for overcoming this difficulty.

(1) *Dilution of reactants*

The antigen and antibody solutions are allowed to diffuse through the gel for a certain period and interact. The areas under any observable peaks as in Pl. 1, fig. 1, are measured and the quantities present calculated. By diluting the antigen and antibody solutions to the same extent any dense bands will diminish and eventually give measurable peak areas. The positions of the bands should be unchanged as both the antigen and antibody solutions are diluted to the same extent. From the measured areas under the peaks at different dilutions the quantity of each antigen-antibody complex can be calculated.

(2) *Dissolving the precipitates*

Turner & Boyer (1952) have shown that the antigen-antibody precipitates may be completely dissolved at pH 11 or above, forming large aggregates with particle sizes about × 250 the diphtheria antitoxin molecule in the case of the diphtheria toxin-antitoxin complex. Some loss of serological activity occurs but this should have little effect, if any, on the refractive index of the proteins concerned. Kleinschmidt & Boyer (1952) found that a solution of sodium dodecyl sulphate was the most active reagent for dissolving the complexes and this was found to be true also for the complexes formed in the gels. Unfortunately, sodium dodecyl sulphate dissolves gelatin most effectively, so this reagent can only be used with agar gel. It was found, however, that provided the pH was kept below 13 units and the gelatin was not too weak the bands of precipitate could be dissolved without dissolving the gelatin. Both sodium dodecyl sulphate and alkaline solutions may be used for dissolving bands of precipitate formed in agar. Two techniques were developed for dissolving the bands.

(*a*) *By diffusion of reagent from the antigen or antibody wells*

This could be applied to both gelatin and agar columns. The wells at either end of the diffusion column were drained and filled with the reagent. The reagent slowly diffused and dissolved the bands of precipitate. The stages in the dissolution of an opaque band of precipitate with the formation of a peak is illustrated in Pl. 2, figs. 3–6. The peaks gradually become broader and flatter as the particles of complex diffuse away. This process may take from a few minutes to several hours depending on the strength of the reagent at the site, and the nature and quantity of the precipitate present. Unfortunately the presence of a diffusion gradient of the reagent can sometimes obscure the peaks obtained and to try to avoid this the second technique was developed.

(*b*) *By diffusion of the reagent from the side of the diffusion column*

This technique has only been tried with agar gel owing to the difficulties encountered in exposing the side of a gelatin column without distortion of the gel and hence of the fringes. The glass cell is split with the blade of a scalpel and the column of gel is floated off the microscope slide into a solution of the reagent, and then quickly placed between the interference plates, the air gap between the column of gel and the plates being filled with a thin film of reagent solution. Effective diffusion appears to take place mainly from the exposed surfaces where the gel is in contact with a large volume of reagent solution. The bands dissolve as illustrated in Pl. 2, figs. 1, 2. The advantage of this technique over the first one is that all bands are dissolved simultaneously, the reagent diffusion gradient being across the width of the cell instead of along its length and thus each band should be dissolved in the same gradient and any distortions are the same for each peak obtained. The disadvantage is that the peaks appear to be broader and flatter than those obtained by the first technique.

Both these methods possess an advantage over the dilution of reactants technique in that it is possible to obtain a measure of the quantities present in each band using a single diffusion cell, which need only contain 0·1–0·2 ml. of antigen and antibody solution. Generally, weak bands of precipitate are obliterated before the transient peak areas can be measured, and in practice it is preferable to combine both the dilution and dissolution techniques, i.e. measure the areas under the peaks where the precipitate is not dense enough to interfere, then dissolve the bands and estimate the quantity present in each of the peaks resulting from the dense bands. In order to determine the total mass of complex in a given band the displacement of the fringes in a direction perpendicular to the length of the channel should be measured, the fringes in the clear medium being set to run parallel to the length of the channel.

Let us consider a particular point in the reaction zone (distance l cm. along the channel) where the shift relative to the clear medium is n_e fringes (expressed in whole fringes plus any fractional shift). The retardation of the light beam is then equal to $n_e\lambda$ cm., where λ is the wave-length of mercury green radiation. Suppose that the refractive index of the medium at this point is equal to μ_e while that of the clear medium is μ_m. If t cm. is the cell depth

$$2t\,(\mu_e-\mu_m)=n_e\lambda.$$

The specific protein increment (α) is the increase of refractive index for the addition of 1 g. of protein to 100 ml. of medium (0·00185 for most proteins). If x g./100 ml. is the concentration of complex at the given point in the reaction zone

$$x=\frac{\mu_e-\mu_m}{\alpha}.$$

Suppose that the width of the channel is w cm. and that the extreme edges of the zone of precipitation are at points l_1 and l_2 along the length of the channel.

$$\begin{aligned}\text{Total mass in the reaction zone} &= \int_{l_1}^{l_2} \left(\frac{\mu_e - \mu_m}{\alpha}\right) tw\,dl \\ &= \int_{l_1}^{l_2} \frac{n_e \lambda}{\alpha 2t}\, tw\,dl \\ &= \frac{\lambda w}{2\alpha} \int_{l_1}^{l_2} n_e\,dl.\end{aligned}$$

$\int_{l_1}^{l_2} n_e\,dl$ is simply the area measured under the peak expressed in fringe displacement along the length of the channel.

A measurement of this quantity indicates the total mass of antigen-antibody complex formed within the reaction zone.

There are errors inherent in both these procedures, but they should be less than those mentioned by Becker (1953) in estimations by Nordstrom in his laboratory of bovine serum albumin with rabbit anti-bovine serum albumin using the Oudin technique, when errors of 50–60% were encountered.

GENERAL OBSERVATIONS

We have obtained indications that when a band of precipitate becomes too dense it can act as a barrier, since the concentration of substances in solution on one side of the band has been found to be higher than on the other side. It should be emphasized that this has only been observed in a few extreme cases. It is probable that beyond a certain critical density the band of precipitate may become virtually impermeable to components of very high molecular weight. Similarly, with very dense bands the structure of the supporting gel appears to be disrupted where the band is formed since the column of gel will break exactly at that point if gently agitated in water. With care the thin sheet of precipitate may be separated quite sharply from the gel on either side by this treatment.

The interferometric observations are in agreement with the principle of Goldberg (1952) that the aggregates of antigen and antibody will continue to grow up to a critical point at which a few large aggregates will be formed by the union of numerous aggregates of moderate size, which then precipitate. Most complexes may be detected as peaks in the interference fringes before any precipitate is formed. These peaks do not diffuse away if the antigen and antibody solutions are withdrawn, and must therefore be in the form of aggregates too large to diffuse through the interstices of the gel but not sufficiently large to form a visible precipitate.

The examination of the diffusion gradients of the antigen or antibody solutions alone in the absence of the other reactant has not revealed any indication of the absorption of any component by the gel, whether agar or gelatin. Diffusion is much slower through gelatin and the resulting bands and peaks appreciably sharper. In using the double-diffusion technique it has also been observed that if the antigen and antibody wells are at least three to four times larger in volume than the column of gel between them, the bands of precipitate and peaks remain stationary once they are formed indicating that a steady state has been formed.

It is hoped that the technique may prove of general application, particularly to a study of the complex mixtures of proteins associated with problems of tissue specificity.

SUMMARY

1. A sensitive interferometric method of observing antigen-antibody complexes formed by diffusion of the reactants through gels in suitable diffusion cells is described.

2. Bands of antigen-antibody complex, if not too dense, are observed as peaks in the interference fringes, the areas under the peaks being proportional to the quantities present in each band.

3. The method may be made quantitative by measurement of the areas under the peaks of bands in which precipitation has not progressed too far, and by dissolving the bands of dense precipitate by diffusion of a suitable reagent to give peaks in the fringes.

4. An equation expressing the quantity of precipitate present in a band has been derived.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Diphtheria toxin-antitoxin system in the interferometer, showing interference fringes and one opaque band of precipitate; also a number of peaks where precipitation either has not occurred or has just commenced.

Fig. 2. Another diphtheria toxin-antitoxin system in the interferometer with the interference fringes almost completely obscured so that only visible bands of precipitates are detectable.

Fig. 3. A rat serum-antirat serum system showing numerous small peaks indicating the presence of many complexes, most of which had not formed any visible precipitate. The group of fine dark bands on the right-hand side show the multiplicity of a band of precipitate which appeared single under the ordinary microscope.

Fig. 4. A greatly enlarged photograph of the main band from the diphtheria toxin-antitoxin system taken with the interferometer using a large pinhole and the fringes almost completely obscured. Six or more components may be seen. This effect could only be detected at an early stage in the development of the band and with a considerable distance between the toxin and antitoxin wells.

PLATE 2

Fig. 1. Opaque band of precipitates before dissolution.

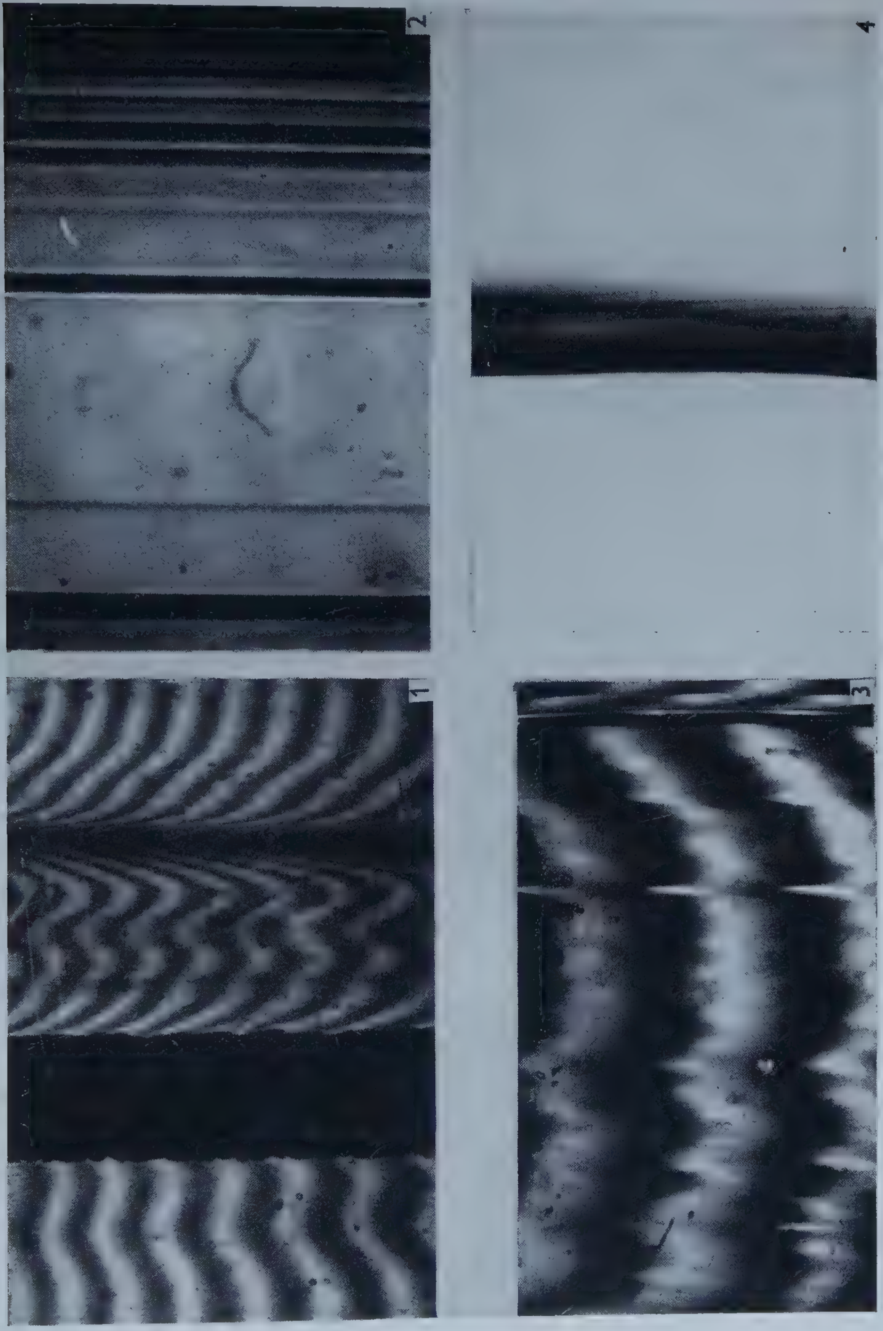
Fig. 2. Same band showing fringes and formation of a peak on dissolution of the band of precipitate by diffusing reagent into the side of the column of gel. In the photograph diffusion is taking place from the upper edge.

Fig. 3. A band of precipitate showing fringes before dissolution of the precipitate.

Fig. 4. The same band with reagent diffusing from the left. The opaque band has almost completely dissolved producing a marked inflexion of the fringes.

Fig. 5. The same band 5 min. later. The band has completely dissolved and a single fringe may be followed through from one side of the peak to the other.

Fig. 6. The same band 10 min. after fig. 5. The peak is shorter and broader as diffusion of the dissolved and dissociated antigen-antibody complex proceeds.



EASTY AND AMBROSE—AN INTERFEROMETRIC METHOD OF QUANTITATIVE ANALYSIS OF ANTIGEN-ANTIBODY REACTIONS IN GELS

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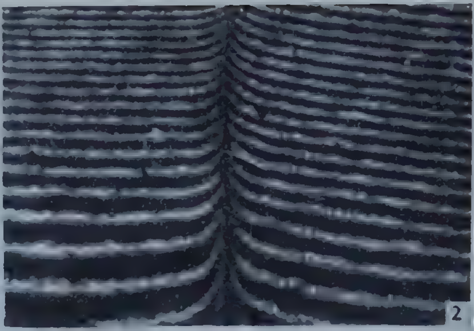

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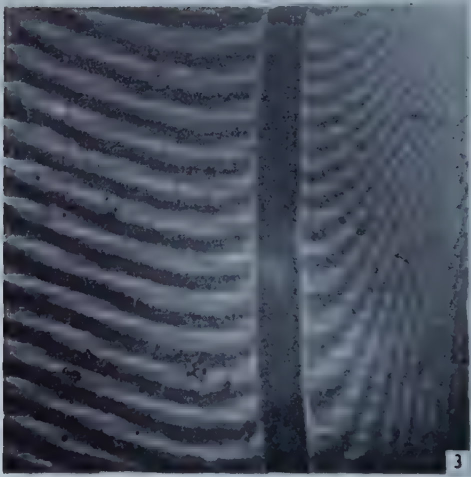

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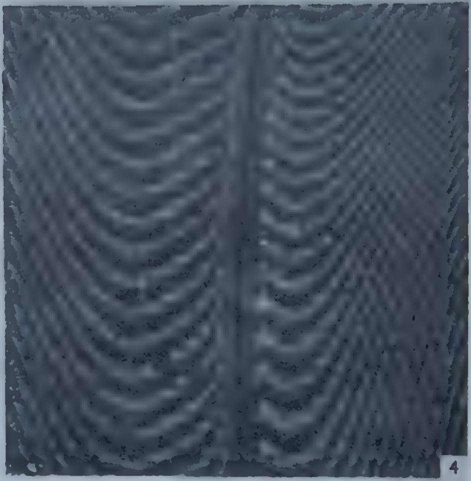

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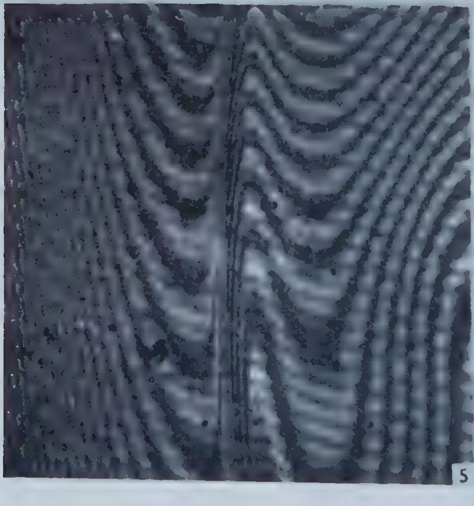

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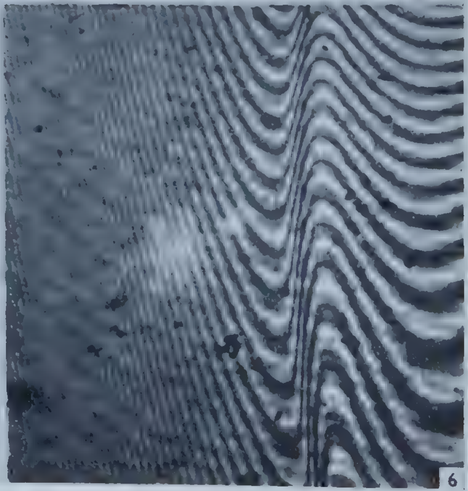

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EASTY AND AMBROSE—AN INTERFEROMETRIC METHOD OF QUANTITATIVE ANALYSIS OF ANTIGEN-ANTIBODY REACTIONS IN GELS

IONIC REGULATION IN THE CILIATE *SPIROSTOMUM AMBIGUUM*

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It is generally accepted that the cytoplasm of fresh-water Protozoa is hypertonic to the medium. This is based on circumstantial evidence and indirect measurements of the osmotic pressure of the body fluids. Inorganic ions are likely to account for a large part of the osmotic pressure, and in certain Protozoa radioactive isotopes may be used to determine the concentrations of ions. The isotope technique has been used to demonstrate that cells in Metazoa, namely nerve, muscle and erythrocytes, accumulate ions and carry out ionic regulation. Evidence is presented in this paper concerning the tonicity of the body fluids of the heterotrichous ciliate *Spirostomum ambiguum* and concerning its powers of ionic regulation.

The concentration of sodium, potassium and bromide have been measured in animals grown in medium containing these ions. The rates of entry and loss of these ions have been measured and the results are discussed in the light of current theories of the mechanisms of ionic regulation in other cells.

MATERIALS AND METHODS

Spirostomum ambiguum was chosen for the experiments as it is easily cultured, it is relatively large and it survives the experimental conditions. It was cultured in the following medium:

	mM/l.
Potassium chloride	0·5
Sodium chloride	2·0
Magnesium chloride	0·2
Calcium chloride	0·5
Potassium dihydrogen phosphate	0·1 (approx)
Potassium hydroxide	0·01 ,,

The medium was made up with glass distilled water and the pH was adjusted to 6·3 by addition of the KH_2PO_4–KOH buffer. Boiled wheat grains were added as a source of food for the bacteria on which *Spirostomum* feeds.

^{42}K was obtained as the 'Specpure' carbonate from A.E.R.E., Harwell, and converted to the chloride by addition of hydrochloric acid; the excess acid being removed by evaporation. When the ^{42}K had decayed the concentration of potassium in the experimental solution was confirmed by measurement with a flame photometer. ^{22}Na was supplied by the Radiochemical Centre, Amersham, as a sodium chloride solution. ^{82}Br was obtained from A.E.R.E., Harwell, as ammonium bromide. The compound was converted to sodium bromide by dissolving it in sodium hydroxide and evaporating the water and ammonia.

In each experiment approximately 50,000 animals were removed from the cultures and concentrated in a small volume of medium by centrifuging at approximately 15 g. for 1 min. The animals were then kept for 3 days in unlabelled medium under experimental conditions to come into a steady state before being transferred to a similar but labelled medium. Several samples of the radioactive medium were taken with an 'Alga' pipette for determination of the radioactivity per unit concentration of medium. At suitable times samples of several hundred animals were taken from the labelled solution. These animals were washed free of labelled solution by centrifuging with six changes of unlabelled medium. The time taken for washing was recorded so that correction could be made for the washing of labelled ions out of the animals. From the last wash the animals were transferred to a planchette with a 0·1 ml. pipette operated by a hypodermic syringe. A sample of the last washing medium was also taken with the same pipette to enable correction to be made for any residual radioactivity of the medium on the animals The count of this sample was never more than one or two counts above the background count; it was used to correct the sample for background count and that of contaminating medium. The animals on the planchette were fixed and stained with a few drops of saturated aqueous picric acid and photographed. Photographing the animals made it possible to carry out the slow process of counting them after all the samples had been assayed for radioactivity. This permitted many more samples to be taken during the useful life of the isotope. The planchettes were dried in an oven at 100° C. before being assayed for radioactivity.

The radioactivity was assayed with a Geiger–Muller end-window counter. The time in half seconds for 1000 counts was recorded for all samples, so that the standard deviation of any count is approximately 3% of the total count. In experiments with ^{42}K and ^{82}Br the counts were corrected for decay to a zero time by calculation using the measured half-lives of the isotopes.

The calculation of the mean volume of a population of animals was based on measurements of photographs of a representative sample of contracted animals. A sample of animals was concentrated into a small volume of medium and transferred to a microscope slide which was held in position with a circle of paraffin wax. When a condenser was discharged through the drop via two electrodes embedded in the wax, the animals contracted. Photomicrographs of the contracted animals were taken. The animal appears as an ellipse on the photograph, and assuming that it is a prolate spheroid the volume may be calculated as $\frac{4}{3}\pi ab^2$, where a and b are major and minor semi-axes. A micrometer scale was photographed with each sample.

THEORETICAL CONSIDERATIONS

In the experiments to be described the animals were transferred from one medium in which they had reached a steady state to another of the same composition but containing labelled ions. The tracer ion then exchanges with its counterpart inside the animal and eventually the specific activity of the tracer inside the animal becomes the same as that of the medium. There is no evidence to show that tracer ions behave differently from the normal species. With a knowledge of the specific activity of the tracer in the medium, the radioactivity when it has reached a steady state in the animal and the amount of body water, the internal concentration may be calculated. This value does not necessarily correspond to the total concentration of the ion inside the animal; only material which will exchange with the labelled ion is measured. Morphological or chemical isolation of the ion inside the animal would give a measured value below the true value.

The equation from which transfer constants may be calculated for the entry of labelled ions into a single cell is given by Davson (1951) as

$$\frac{d\,C_{\text{in}}}{dt} = K_{\text{in}}\,C_{\text{out}} - K_{\text{out}}\,C_{\text{in}}. \tag{1}$$

K_{in} is the transfer constant from out to in and has dimensions of hours^{-1}. K_{out} is the transfer constant from in to out and also has the dimensions of hours^{-1}. t is the time in hours during which the animals are in the labelled solution. C_{in} is the concentration of the labelled ions in the cell. C_{out} is the concentration of labelled ions in the medium and may be regarded as constant when the volume of medium is very large compared with that of the animals.

As there is no net change of concentration of labelled ions when the steady state is reached it follows that

$$\frac{C_{\text{in}}}{C_{\text{out}}} = \frac{K_{\text{in}}}{K_{\text{out}}}. \tag{2}$$

The solution of equation (1) for K_{out}, in the case of animals in labelled medium of constant concentration, is

$$-K_{\text{out}}\,t = \ln\left(1 - \frac{C_{\text{in}(t)}}{C_{\text{in}(t=\infty)}}\right), \tag{3}$$

where t is the time the animals are in the radioactive solution. For practical purposes $C_{\text{in}\,(t=\infty)}$ may be regarded as a steady value obtained at finite time.

In the case of animals which have taken up labelled ions and are then transferred to a similar but unlabelled medium, the solution of equation (1) is

$$-K_{\text{out}}\,t = \ln\left(\frac{C_{\text{in}(t)}}{C_{\text{in}(t=0)}}\right), \tag{4}$$

where t is the time in the unlabelled solution. As the ratios of the values of C_{in} are used in equations (3) and (4), the units in which they are measured are not important and for convenience counts per animal per minute are used in the calculation of K_{out}.

The experimental data when plotted according to equations (3) or (4) should result in straight lines if the animal behaves as a single-compartment system. The value of K_{out}, as measured, is not an absolute transfer constant as it is dependent on the size of the animal. K_{out} is related to the absolute rate constant K'_{out} by the equation

$$K_{\text{out}} = K'_{\text{out}}\,\frac{A}{V}, \tag{5}$$

where K'_{out} is the absolute rate constant as cm. hours^{-1}, V is the volume of the animal in cm.^{3} and A is the surface area in cm.^{2}.

EXPERIMENTAL RESULTS

Experiments with ^{42}K

Uptake

In the first experiment the level of potassium was measured over a period of 55 hr. in animals kept at room temperature. Results of this experiment are shown in fig. 1, where the count per animal per minute is plotted against time in the radioactive solution. In all experiments each point is the mean activity determined for 50–500 animals, and all samples at a given time were washed together. The scale on the right of the graph indicates the calculated concentration of potassium in the animal on the assumption (to be discussed later) that the animal is 100% water.

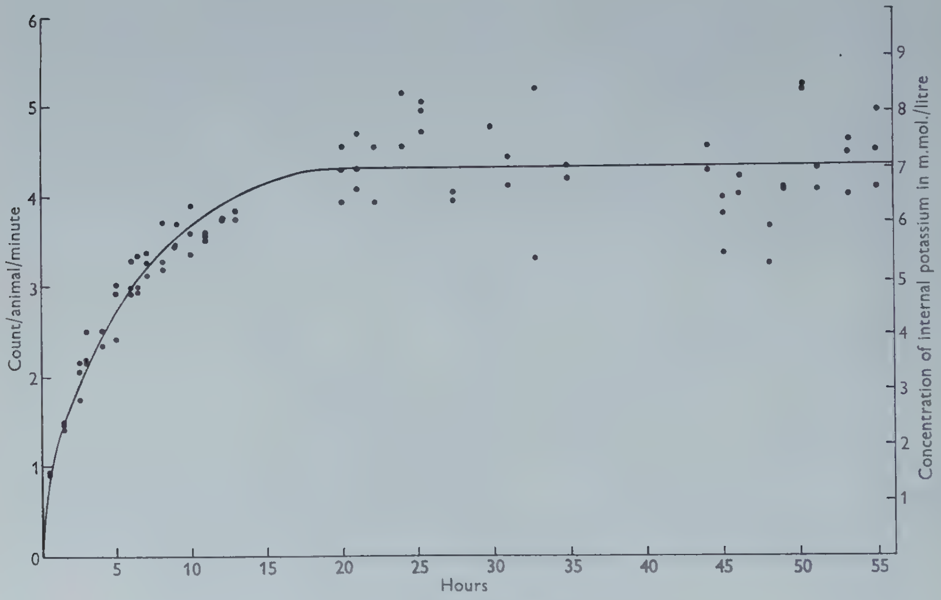

Fig. 1. The uptake of ^{42}K by *Spirostomum* from culture medium.

In Table 1 the results of several similar experiments are summarized. The level of activity in the animal remains fairly constant during an experiment, but from column 3 of Table 1 it may be observed that the mean volume frequently falls off with time. Assuming that the mean volume alters at a constant rate, calculations of mean volumes have been made to obtain the best estimates at the beginning and end of the period during which the level of activity in the animal was measured. The calculated concentrations based on these volumes are shown in column 5 of Table 1. The means of the levels of the radioactivity in the animals during the period stated are listed in column 4 of Table 1 and they are corrected for loss during the washing period from data obtained in Exp. 4.

It is clear from Table 1 that the potassium concentration inside the animal is at least ten times that in the medium.

Table 1

2 External potassium concentration (mM/l.)	3 Volume		4 Activity		5 Internal potassium concentration (mM/l.)
	Time measured (hr.)	Mean volume ± S.E. of mean (mm.^{3} × 10^{-4})	Time measured (hr.)	Mean activity ± S.E. of mean (counts/animal/min.)	
0·475	0	59 ± 6·5	20 to 55	4·4 ± 0·05	6·2
	70	37 ± 4·1			7·9
0·48	12	74 ± 4·7	20 to 32	17·3 ± 0·09	5·5
	33	62 ± 2·5			6·1
0·48	0	40 ± 1·6	18 to 30	10·3	8·5
	72	28 ± 1·3			9·0
0·49	0	47 ± 1·4	20 to 72	5·9	6·9
	72	48 ± 1·4			7·1
0·1	73	36 ± 1·4	12 to 38	9·1 ± 0·1	8·9
	73	28 ± 1·2	40 to 66	8·2 ± 0·1	10·3
	73	27 ± 1·6	24 to 34	8·4 ± 0·2	10·9
0·07	56	32 ± 0·4	10 to 51	8·9 ± 0·1	7·0
0·42	0	65 ± 8·6	22 to 54	19·2 ± 0·5	4·5
	44	65 ± 2·6			
0·50	5	52 ± 2·5	8 to 12	19·5 ± 0·3	7·6
	20	59 ± 3·1			6·6

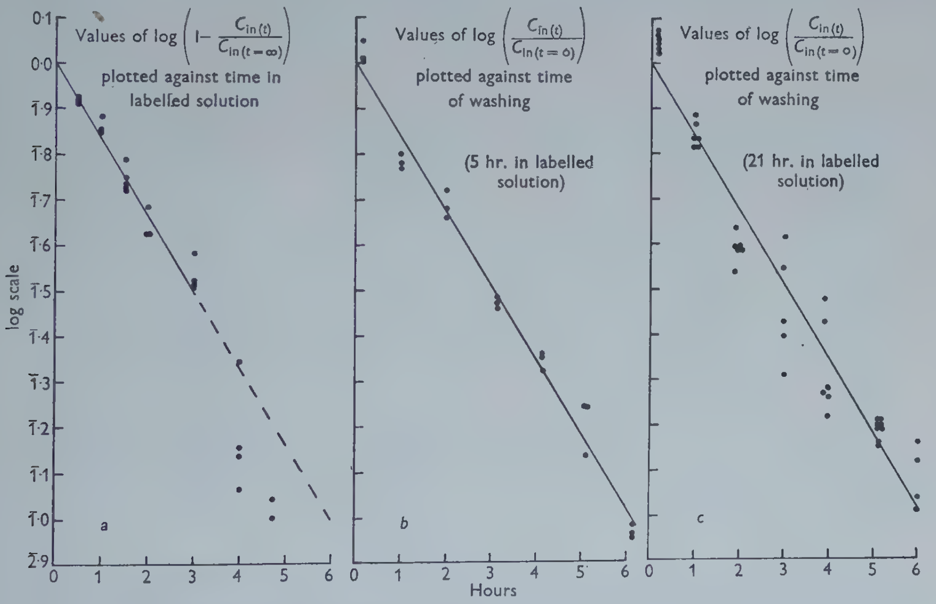

Fig. 2. The uptake and loss of ^{42}K from *Spirostomum* plotted according to equations (3) and (4).

Measurements of the transfer constants K_{in} and K_{out}

To obtain data for plotting according to equations (3) and (4), the rate of uptake of potassium was measured in a population of animals, and when the animals had taken up an appreciable amount of labelled ions they were washed for variable periods in unlabelled medium and the rate of loss measured. The values of

$\log\left(1-\frac{C_{\text{in}(t)}}{C_{\text{in}(t=\infty)}}\right)$ were calculated from the uptake data and they are plotted against time in Fig. 2*a*. The value of $C_{\text{in}(t=\infty)}$ is the mean of all measurements made whilst the animals were in a steady state with the medium. As $C_{\text{in}(t)}$ approaches $C_{\text{in}(t=\infty)}$ variation becomes greater, and as Solomon (1952) pointed out only the first few points describe a straight line. Following Treherne (1954) the line drawn through the data is the mean of the slopes calculated for lines adjoining the individual points to the origin. As there is a marked change of slope after 3 hr., only data for this first period are considered. The slope of this line is $-K_{\text{out}}/2\cdot303$.

The rate of loss of labelled ions from the animals was measured after 5 hr. and after 21 hr. in the labelled solution, that is, prior to and after reaching a steady state with respect to the labelled ion. The data plotted according to equation (4) are shown in Figs. 2*b* and *c*. The slopes of the regression lines through the data are equal to $-K_{\text{out}}/2\cdot303$.

The slopes of the lines in Figs. 2*a*, *b* and *c* are practically the same, and so it follows that the values of K_{out} measured by several methods must be almost identical. This, together with the results of other experiments listed in Table 2, is considered to be justification for the theoretical treatment and it may be concluded that *Spirostomum* behaves as a one-compartment system to potassium.

Table 2

Exp. no.	External potassium concentration (mM/l.)	Mean volume ± S.E. of mean (mm.^{3} × 10^{-4})	Temp. (° C.)	Equation used for calculation of K_{out}	K_{out} (hours^{-1})	C_{in} (mM/l.)	K_{in} (hours
1	0·475	48 ± 5·3	16·5	3	0·259	7·05	3·9
2	0·48	68 ± 3·4	18	3	0·317	5·7	3·8
				4	0·473		5·5
3	0·48	40 ± 1·6	16·5	4	0·296	8·75	5·4
4	0·49	47 ± 1·4	16·5	4	0·270	7·0	3·8
6	0·07	32 ± 4·0	22	3	0·393	7·0	39·3
12	0·45	91 ± 4·5	18	3	0·263	4·5	2·9
		95 ± 3·8	18	3	0·256	4·0	2·4
13	0·42	65 ± 8·6	18	3	0·29	4·5	3·1
22	0·5	55 ± 2·8	18	3	0·38	7·1	5·5
				4 (5 hr.)	0·38	7·1	5·4
				4 (21 hr.)	0·38	7·1	5·4
8	0·04	51 ± 1·7	18	3	0·35	3·0	38·7
	1·00	42 ± 1·5	18	3	0·27	9·4	3·5

It may be seen from Table 2 that when the concentration of the external medium is altered the values of K_{out} remain relatively constant, but K_{in} varies considerably. The significance of this point will be discussed later.

The effect of feeding on the uptake of potassium

Spirostomum feeds on bacteria which grow in the culture medium when boiled wheat grains are added. To obtain a source of food for these experiments the common bacteria from a thriving *Spirostomum* culture were plated out and grown on agar slopes.

The uptake of potassium was measured in three groups of animals of common stock. Group 1 was fed throughout the experiment, whilst group 2 was fed until it was transferred to the labelled solution and group 3 was starved until it was transferred and then fed. After the initial separation of the animals feeding where necessary was carried out every 12 hr. until 6 hr. before placing the animals in the labelled solution. From then onwards it was carried out every 2 hr. in order to maintain the supply of bacteria in the medium.

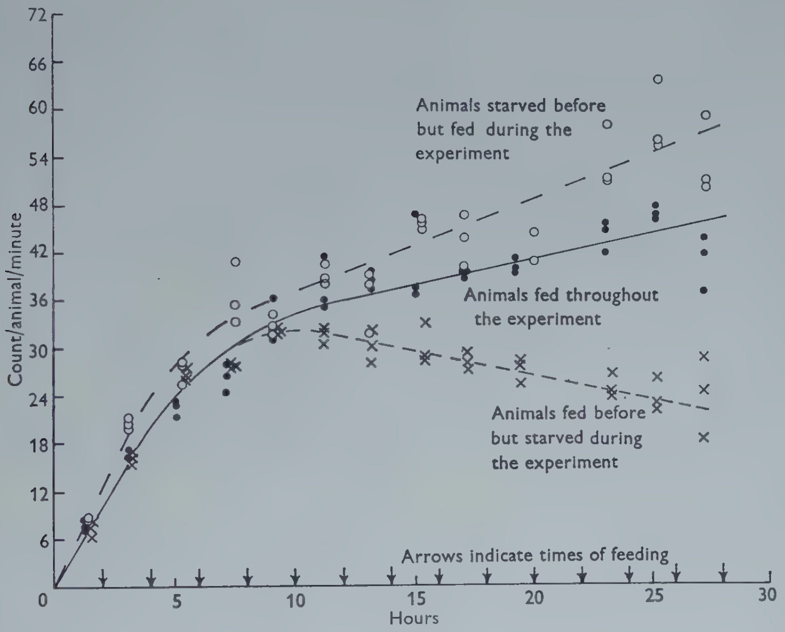

Fig. 3. The effect of feeding and starvation on the uptake of ^{42}K by *Spirostomum.*

The results of the experiment are shown in Fig. 3. The animals of group 1 do not reach a steady state with labelled potassium during the experiment; the value of C_{in} at 28 hr. is calculated as 9·8 mM/l. The animals of group 2 attain a maximum activity at 10 hr., i.e. the normal period, and then the level falls. This fall of activity is associated with a similar fall of volume so that the concentration of internal potassium remains fairly constant at 6·7 mM/l. The potassium activity in animals of group 3 continues to rise throughout the experiment at a greater rate than in animals of group 1. The mean volume of these animals does not appear to alter during the experimental period, and the value of the internal potassium at 27 hr. is 12·5 mM/l. Obviously the potassium concentration is greatly influenced by feeding, and this probably accounts for much of the variation in the experimental results.

The effects of changes of the external potassium concentration

Animals of the same population were acclimatized to culture media containing 0·02, 0·08, 0·32 and 1·28 mM/l. potassium for 3 days. They were then transferred to similar but labelled media, and after 12 hr. the concentrations of potassium in the animals were measured. The means of the volumes of the animals in the various concentrations were practically the same and the internal potassium concentrations were calculated as 3·2, 4·5, 5·7 and 7·8 mM/l. respectively.

It is concluded that the animal is capable of regulating its potassium content even when the external concentration is very low.

The uptake of potassium at different temperatures

The potassium activities were measured in groups of animals of the same population kept at different temperatures. It was found that animals kept at 15° C. contain three times the amount of labelled ions found in animals at 25° C. This difference in activity was associated with a difference in volume so that the potassium concentrations were practically the same. Owing to the volume difference, comparable values of transfer constant were not obtained and a Q_{10} for potassium transport could not be calculated.

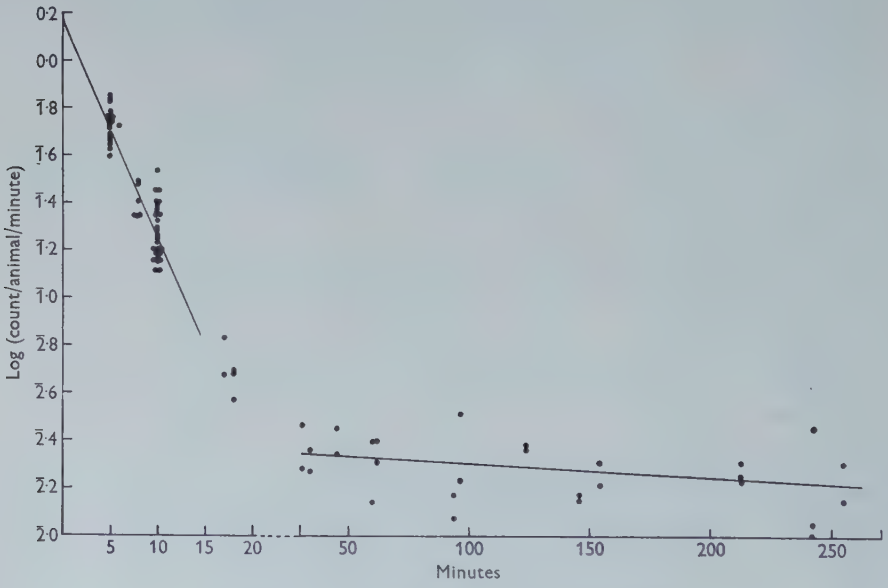

Fig. 4. The rate of loss of ^{22}Na from *Spirostomum*.

Experiments with ^{22}Na

The uptake of sodium and measurements in K_{in} and K_{out}

A preliminary experiment with labelled sodium indicated that the animals reached a steady state with the ion in a matter of minutes and that the apparent internal concentration was very low. Therefore the rate of loss of sodium was measured from animals which were in a steady state with the labelled ion. The logarithms of the activities ($C_{\text{in}(t)}$) measured during Exp. 16 are plotted in Fig. 4 against time in unlabelled medium. It is clear from the figure that the washing out process may be divided into two components: a fast component which is calculated to account for 98% of the sodium in the animal and a slow component which for practical purposes may be disregarded. The animal is considered to behave in the main as a one-compartment system to sodium. The concentration of sodium in the animals shown in Table 3 was calculated from the activity in the animals at zero time which was found from the regression of log C_{in} against time, only the first 10 min. being considered. A plot of this data according to equation (4) is equivalent to altering the scale of the ordinate so that log $C_{\text{in}(t=0)}$ is zero. The slope of the regression line is equal to $-K_{\text{out}}/2{\cdot}303$.

Table 3

All experiments carried out at 18° C.

Exp.	External sodium concentration (mM/l.)	Mean volume ± S.E. of mean (mm³ × 10⁻⁴)	K_{out} (hours⁻¹)	C_{in} (mM/l.)	K_{in} (hours⁻¹)
16	2·0	79 ± 3·8	13·7	1·05	7·2
17	1·0	59 ± 2·2	12·2	1·02	12·4
	2·0	59 ± 2·7	14·0	1·57	11·1
	4·0	66 ± 3·0	14·6	1·62	5·9
18	1·0	62 ± 2·9	15·6	0·83	12·9
	6·0	67 ± 3·5	12·4	0·91	1·9

Two further experiments, 17 and 18, were carried out to determine the values of C_{in} and K_{out} for animals kept in media with different sodium concentrations. Fortunately, the volumes of the animals were not significantly different in the two experiments, so that the transfer constants listed in Table 3 may be compared. There is no correlation between the concentration of the medium and the values of K_{out}, but the values of K_{in}, calculated from equation (2), decrease with increasing C_{out}. Allowing for the variability of the material and experimental error it is considered that C_{in} and K_{out} are constant when C_{out} is varied from 1 to 6 mM/l. The significance of constant values for C_{in} and K_{out}, but a variable K_{in} when C_{out} is altered, will be discussed later.

Experiments with ^{22}Na *and* ^{82}Br

The concentration of sodium and bromide in the animals

Before discussing the significance of the concentration of cations in the animals it is necessary to know the concentrations of the anions. Chloride ions are likely to be the most concentrated inorganic anions in the animal, but it was impracticable to use a radioactive isotope of chlorine for their measurement. As an alternative

^{82}Br was used. Preliminary experiments showed that the animals would live in a culture medium where the sodium and potassium chlorides were replaced by bromides.

The uptake of labelled bromide is illustrated in Fig. 5. The scatter of results at the beginning of the experiment is attributed to contamination of the ^{82}Br by ^{80}Br. The concentration of bromide in the animal is calculated as 0·34 mM/l., whilst in the medium it was 2·5 mM/l. It is clear that the animals maintain a lower bromide concentration in their bodies than is found in the medium.

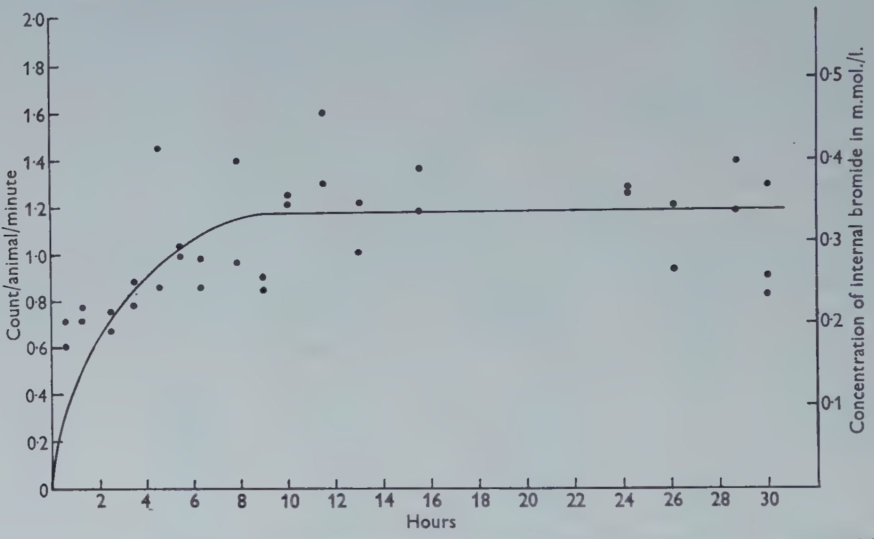

Fig. 5. The uptake of ^{82}Br by *Spirostomum* from culture medium containing 2·5 mM bromide.

Table 4

External concentration		Mean volume ± s.e. of mean (mm^3 × 10^{-4})	Internal concentration		
Sodium (mM/l.)	Bromide (mM/l.)		Sodium (mM/l.)	Bromide (mM/l.)	Halide (mM/l.)
1·19	1·69	53 ± 1·9	1·08	0·30	0·58
2·54	3·04	66 ± 3·5	1·01	0·41	0·58
4·09	4·59	55 ± 3·0	1·49	0·47	0·63

Animals were transferred from culture media to similar but labelled culture solutions containing labelled sodium and labelled bromide at three different concentrations. After 12 hr. samples were taken and washed for periods ranging from 5 min. to 4 hr. and they were assayed for radioactivity before and after the bromide had decayed. It is calculated from the regression of the bromide activity against time that animals in a medium containing 3·0 mM/l. bromide lose 4·3% of the labelled ion during the washing period of 5 min.

The calculated concentrations of sodium and bromide in the animals are summarized in Table 4. On the assumption (to be discussed later) that the proportion of bromide to total halide is the same inside as outside the animal, the concentrations of total halide in the animals have been calculated and are shown in the last column of Table 4. Once again there is no correlation between the sodium concentrations in the animal and those in the medium and the values are similar to those of previous experiments.

Table 5. *The concentrations of inorganic ions in* Spirostomum ambiguum *compared with the medium. All concentrations are expressed as milli-osmoles/litre*

Ion	Medium	Cell	Medium	Cell	Medium	Cell
Na	1·19	1·08	2·54	1·01	4·09	1·49
K	0·65	7·0	0·65	7·0	0·65	7·0
Mg	0·2	?	0·2	?	0·2	?
Ca	0·5	?	0·5	?	0·5	?
Total cations measured	2·54	8·08	3·89	8·01	5·44	8·49
Br	1·69	0·30	3·04	0·41	4·59	0·47
Cl	1·4	0·28	1·4	0·17	1·4	0·16
PO_4	0·1	?	0·1	?	0·1	?
Other anions	0·05	??	0·05	??	0·05	??
Total anions measured	3·24	0·58	4·59	0·58	6·14	0·63
Total ions measured	5·78	8·66	8·48	8·58	11·58	9·12

DISCUSSION

The mean gross volume has been used in calculating the concentrations of ions in the animals as no accurate method is readily available for measuring the water content of *Spirostomum*. Grobicka & Wasilewska (1925) estimated that *Paramecium caudatum* has a water content of 89%, whilst Iida (1940) estimated it as 79%. By analogy it is expected that *Spirostomum* would have a high water content, and this is borne out by a series of histochemical tests which failed to find any appreciable quantities of fat or glycogen present in the experimental animals. It is suggested that the body water is at least 75% of the body volume, so that the calculated ionic concentrations may be subject to a correction not exceeding 33% to allow for the non-aqueous content of the animals.

Even allowing for this correction it is clear that the concentration of bromide is much lower in the cell than in the medium. Experiments with other animals suggest that cells do not discriminate between bromide and chloride ions (e.g. Koch (1938), Krogh (1938), Frey (1937)). Therefore it is assumed that when *Spirostomum* is in a steady state with the labelled ion the concentration of bromide may be used to calculate the total halide content of the cells, but the rate of uptake of bromide is not a measure of the rate of entry of halide.

In table 5 the concentrations of inorganic ions inside the animal are compared with the corresponding concentrations in the media. It may be seen that anions other than halide must be present inside the cells to balance the charges of cations. Conway (1945) suggested that an anion deficit in frog's muscle may be made up by low molecular weight compounds such as creatine phosphate, carnosine and adenosine triphosphate. If similar compounds make up the deficit in *Spirostomum* then it becomes clear that the animals are hypertonic to the medium.

Spirostomum is permeable to potassium ions, and the body concentration which is largely independent of the outside concentration is at least ten times as high as the concentration in the media. The concentration of sodium is much lower than that of potassium, and in media of higher sodium concentration this ion is kept out against the concentration gradient. In common with studies of the distribution of ions in other tissues there is no evidence to show that ion binding is responsible for this unequal distribution of sodium and potassium ions. If, for the purpose of discussion, it is assumed that *Spirostomum* is virtually impermeable to sodium owing to active transport directed outwards, then the high potassium concentration of the cell might be explained by a Donnan equilibrium as proposed by Boyle & Conway (1941) for frog muscle. If the cell is impermeable to sodium, potassium must enter to balance the charges of the indiffusible anions. The conditions are such that

$$\frac{\text{Potassium inside}}{\text{Potassium outside}}=\frac{\text{Halide outside}}{\text{Halide inside}}.$$

The ratios of the concentrations of ions in the cells, calculated from data taken from Table 4, are tabulated below:

$\frac{\text{Potassium inside}}{\text{Potassium outside}}$	10·8	10·8	10·8
$\frac{\text{Halide outside}}{\text{Halide inside}}$	5·3	7·6	9·5

It appears that a Donnan equilibrium might account for the high concentration of potassium and low concentration of halide in the cell if due allowance is made for experimental error and the variability of the animals.

Without a knowledge of the electrical potential gradient across the cell membrane of *Spirostomum* it cannot be conclusively proved that active transport of any ion is taking place, since the only certain criterion, defined by Rosenberg (1948), is that the ion in question is transported against the electrochemical gradient. There is no direct evidence to show that sodium is actively transported, but it does seem likely since otherwise it would be difficult to account for the low content of the cell when the external concentration is high. The fact that the exchange of sodium is much more rapid than that of potassium points to sodium passing through the cell membrane as an undissociated complex, since in the ionic state sodium diffuses more slowly than potassium.

The concentration of sodium in the cell is in some circumstances the same as the medium. Therefore osmotic equality between cell and medium cannot be obtained by opposing concentration gradients of sodium and potassium. However, osmotic influx of water into *Spirostomum* could be counterbalanced by the contractile vacuole pumping out water. Where the concentration of sodium in the medium is high there are opposing concentration gradients so that the osmotic entry of water into the animal will be slowed down.

The values of K_{out} and C_{in} for potassium ions (Table 2) remain relatively constant when C_{out} varies greatly, and as an approximation it may be said that K_{in} varies inversely with C_{out}. K_{in} is a measure of the proportion of the external potassium concentration entering the animal in unit time, so it appears at first sight that an important part of the regulation of potassium is the result of the animals taking it up at the constant rate in spite of changes of the concentration in the medium. Similarly, in the case of sodium ions, C_{in} and K_{out} remain relatively constant when the concentration in the medium is altered (Table 3). The apparent movement of these ions independent of their concentration gradients could readily be explained by a system of exchange diffusion similar to that proposed by Ussing (1947) to account for the rapid sodium exchange in frog muscle. However, exchange diffusion cannot bring about changes of the total amount of any ion in the cell, and since the animals grow and multiply they must take up sodium and potassium. It is unlikely that the concentration of ions could be maintained constant indefinitely without the intervention of some mechanism other than exchange diffusion.

SUMMARY

1. The concentrations of potassium, sodium and bromide in the ciliate *Spirostomum ambiguum* have been measured by equilibration with radioactive media.

2. The potassium concentration in the animal is at least ten times greater than the concentration in the experimental media.

3. At the higher concentrations of the experimental media, sodium is kept out of the animal against the concentration gradient. The rate of uptake and loss of sodium is largely independent of the external concentration.

4. The time of half exchange of cell sodium is approximately 3 min., whereas for potassium it is between 2 and 3 hr.

5. After equilibration of the animals in culture medium containing bromide the cell concentration of bromide is found to be lower than the external concentration.

6. The effects of temperature changes on the uptake of potassium have been investigated. The size of the animal decreases with increased temperature but the internal potassium concentration remains fairly constant.

7. Feeding the animals increases the rate of uptake and the internal concentration of potassium.

8. It is concluded that the cytoplasm of *Spirostomum* is hypertonic to the medium.

9. The experimental results for cation exchange might largely be explained by exchange diffusion, but actively regulating processes seem necessary to account for the maintenance of constant internal concentrations when the animals grow and multiply.

10. It is suggested that potassium is concentrated in the cell in accordance with a Donnan equilibrium arising from the presence of indiffusible anions in the cell and an active process which keeps out sodium.

I would like to thank Dr J. A. Kitching, under whose supervision this work was carried out, for his advice and encouragement, and Prof. J. E. Harris for his active interest in the problem.

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TEMPERATURE TOLERANCE AND ACCLIMATIZATION IN *DROSOPHILA SUBOBSCURA*

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INTRODUCTION

In the course of a study of hybrid vigour in *Drosophila subobscura* it was desired to investigate the effects of inbreeding and of outcrossing on a character which can undergo adaptive modification during development. Since it was known from the work of Mellanby (1939, 1954) that some insects can become acclimatized to changes in temperature, it was decided to study this capacity in *D. subobscura*. It was found that acclimatization does take place, and that its extent is greater in outbred than in inbred flies (Maynard Smith, 1956). In the course of this investigation some results were obtained of interest to physiologists rather than to geneticists, and these are reported here.

Drosophila has certain advantages as a subject for research in insect physiology. First, a number of inbred lines exist, and by crossing such lines vigorous and phenotypically uniform first generation hybrids are obtained. Using such hybrids, variations due to genetic differences between individuals can be reduced, with a corresponding gain in the accuracy with which the effects of changed environmental conditions can be measured. Secondly, the generation time is short (about one month in *subobscura*), and the methods of laboratory culture have been improved over many years, so that it is relatively easy to determine the influences of changed conditions at various stages of the life cycle.

It has therefore been possible to obtain rather accurate estimates of the time for which flies can survive at various temperatures in dry and in saturated air, and to study the reversibility of the changes occurring at high temperatures. The most striking feature of these results has been the very high temperature coefficients obtained for the processes causing death both in dry and in wet air.

Acclimatization has been found to take two forms. The survival time at high temperatures depends not only on the temperature at which adults have been kept immediately before testing, but also on the temperature at which they were raised during pre-adult life. Thus the temperature during pre-adult life has a long-lasting effect on the capacity of adults to withstand exposure to high temperatures.

METHODS

All flies used were F_1 hybrids between two inbred lines, **B** and **K** (Maynard Smith, Clarke & Hollingsworth, 1955). They were raised in half-pint milk bottles on a medium of maize meal, agar and molasses, with living yeast suspension added.

Unless otherwise stated, they were kept at 20° C. until testing, which was carried out from 5 to 9 days after emergence. Flies were removed from the culture bottles on the day of emergence and kept subsequently in vials containing fresh food.

Measurements were made of the length of time for which flies could survive at a constant high temperature, either in dry or in saturated air. Air was supplied by an electric pump at a rate of approximately 0·2 l./min., dried by passing through four columns of anhydrous calcium chloride and then heated in a coiled copper tube immersed in a water tank. The air was then passed in series through seven $3\frac{1}{2}$ by $1\frac{1}{4}$ in. diameter vials, which were also immersed in the tank. The first and last vials contained thermometers, and a third thermometer recorded the temperature of the water. Flies were placed in the last six vials, a single vial usually containing two flies of the same sex. A piece of filter paper was fitted to the bottom of each vial, since it was found that flies which fall on their backs on a glass surface cannot easily right themselves. These filter papers were soaked in a solution of cobalt chloride to indicate the dryness of the air.

For tests in wet air, the air was passed direct to the heating tube and then bubbled via a porous plug through water at the required temperature, contained in a beaker immersed in the tank. Air collected over the water in this beaker was then passed to the vials.

Flies to be tested were transferred to the vials without etherization, the vials were attached to a wooden rack with rubber bands and were then connected in series with rubber tubing. It was then possible to start an experiment in a few seconds by connecting a rubber tube from the heating coil to the first vial and lowering the rack into the tank.

The air temperature in the vials was kept within 0·1° C. of the required value by running hot or cold water into the tank as required. At the start of a test the water was raised to 1·5° C. above the required temperature, and cooled by running in cold water when the air inside the vials reached the required value; in this way, the time from immersing the vials to reaching the required temperature could be reduced to about 10 min.

The vials were mounted so that the flies could easily be watched. At the start of a test, flies are very active. After about one-third of the total time to death, some flies repeatedly fall on their backs and lie supine for several minutes, after which, either spontaneously or because the vial is tapped, they rise and are able to walk about. This period is followed by one in which all the flies stand stationary at the bottom of the vials, occasionally taking a few steps. Finally, the flies fall on their backs and remain there, showing no signs of life if the vial is shaken. Accordingly, all vials were shaken at 5 min. intervals and flies were recorded as dead if they remained on their backs with no further sign of movement. The time of survival of an individual fly was then taken as lasting from the moment when the air temperature reached the required value to the time when death was recorded.

SURVIVAL TIMES IN DRY AND IN SATURATED AIR AT DIFFERENT TEMPERATURES

Fig. 1 shows the survival times of flies in dry and in saturated air plotted against temperature. Each point in the figure is the mean value for six males or for six females. There was little variation between flies of a given sex in given conditions; the coefficient of variation of six such observations varied from 0·03 to 0·30, with a mean value of 0·15. The latter value implies that an estimated mean survival time of, say, 100 min. has a standard error of ± 6 min.

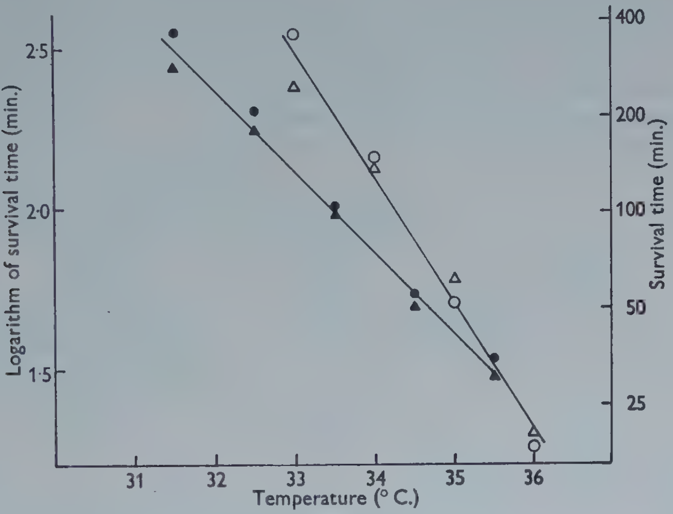

Fig. 1. Survival time of flies in dry and in saturated air at different temperatures. ▲, males, ●, females, in dry air; △, males, ○, females, in saturated air.

In both dry and saturated air there is a linear relationship between the logarithm of the survival time and the temperature. Regression lines fitted to the data for the sexes combined have the equations

$$\left.\begin{aligned} \log_{10} t &= 10{\cdot}54 - 0{\cdot}255\,T \quad \text{in dry air,} \\ \text{and} \qquad \log_{10} t &= 15{\cdot}67 - 0{\cdot}399\,T \quad \text{in wet air,} \end{aligned}\right\} \tag{1}$$

where t = survival time in minutes, and T = temperature in degrees Centigrade.

Sex differences are small, except at the lowest temperatures with the longest survival times. The only significant departures from linearity occurred at 31·5° C. in dry air, and at 33·0° C. in wet air, in both of which conditions the survival times of females continued to fit the linear relationship, whereas the survival times of males were significantly shorter.

These curves suggested that death finally results from the cumulative effects of some process whose rate, v, is proportional to the reciprocal of the survival time, t. If v, v_{T+10} are the rates of this process at two temperatures differing by 10° C., then equations (1) can be rewritten in the form

$$v_{T+10} = Q_{10} v_T, \tag{2}$$

where the temperature coefficient, Q_{10}, has the values $10^{2\cdot55} \simeq 350$ in dry air, and $10^{3\cdot99} \simeq 10{,}000$ in wet air.

These values are startlingly high when compared to the values of from 2 to 3 commonly found for chemical reactions, or from 2 to 6 for metabolic processes. However, the data are so well fitted by a relationship of this kind that it is natural so to describe them, and to suggest that the processes resulting in death at high temperatures must have remarkably high temperature coefficients.

ACCLIMATIZATION TO HIGH TEMPERATURES

Acclimatization to hot dry air

Tests were made on five groups of flies, each consisting of six males and six females, which had been raised at the following temperatures:

Group	Temperature during egg, larval and pupal life	Temperature at which adults were kept from emergence to testing
15–15	15° C.	15° C.
15–25	15° C.	25° C.
15–25–15	15° C.	25° C. for 4 days after emergence, and then at 15° C. for 2 further days before testing
25–15	25° C.	15° C.
25–25	25° C.	25° C.

The period from emergence to testing varied from 5 to 8 days at 15 °C., and from 3 to 5 days at 25° C. Flies were transferred to fresh food vials on the day of emergence, and again on the day before testing.

Survival times were then measured at 33·5 °C. in dry air; the results are shown in Fig. 2.

Flies which had been kept at 25° C. either in the pre-adult or adult stages survived for approximately 2·5 times as long as did the 15–15 group. Individuals kept at 25° C. throughout survived for longer than did those kept at 25° C. for only one period of their lives, but not for as long as would be expected if the increments due to acclimatization as a larva and as an adult were additive.

The acclimatization which occurs in individuals kept at 25° C. during the pre-adult stage is long-lasting, since the survival time was measured after the adults had been kept at 15° C. for about a week. In contrast, the acclimatization during adult life is of short duration; the 15–25–15 group survived only slightly longer than did the 15–15 group, so that the acclimatization which occurred in adults kept for 4 days at 25° C. had largely disappeared after 2 further days at 15° C.

Therefore two kinds of acclimatization can be recognized. The long-lasting changes occurring during pre-adult life will be referred to as 'developmental acclimatization', and the more transitory changes in adults as 'physiological acclimatization'.

It has been assumed above that the observed differences in survival times of different groups are due to processes of acclimatization occurring in individuals, and not to selection acting through differential mortality. The reasons for believing this assumption to be true are given by Maynard Smith (1956).

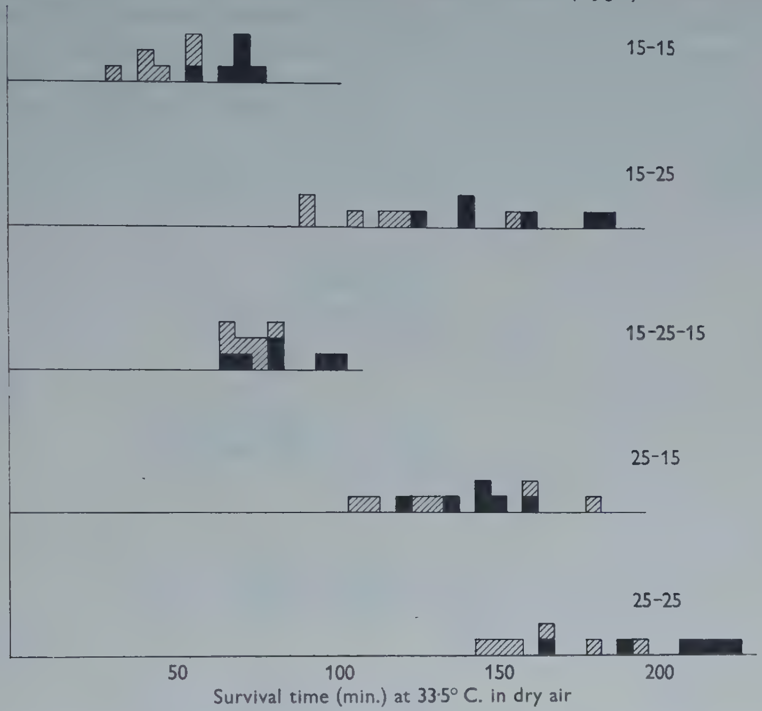

Fig. 2. Survival time in dry air of flies previously kept at different temperatures. Each square represents one individual: black, females; hatched, males. 15–15, individuals kept at 15° C. throughout; 15–25, adults kept at 25° C.; 15–25–15, adults kept for 4 days at 25° C. and then for 2 days at 15° C.; 25–15, pre-adult stages kept at 25° C.; 25–25, individuals kept at 25° C. throughout.

Acclimatization to hot saturated air

A similar series of experiments was carried out on changes in the capacity of individuals to survive in saturated air. Flies belonging to the 15–15, 15–25, 25–15 and 25–25 groups were tested at 34·3° C. in saturated air; this temperature was chosen because, for flies kept at 20° C. throughout, it gives the same survival time as does 33·5° C. in dry air.

The results are shown in Fig. 3. The extent of acclimatization, either in pre-adult or in adult life, although statistically significant, is very small compared to that demonstrated by testing in dry air.

The conditions for physiological acclimatization

The above results show that the changes produced in individuals by keeping them at 25° C. are such as to increase their resistance to desiccation at high temperatures rather than to high temperatures as such. This suggested that the changes might have been produced by exposing individuals to a higher saturation deficiency prior to testing, rather than by the direct effects of a high temperature, since the air in culture bottles and vials kept at 25° C. has a higher saturation deficiency, and probably a lower relative humidity, than that in bottles and vials at 15° C.

This possibility has been investigated only for the processes of physiological acclimatization. Individuals were raised in culture bottles in the usual manner at 20° C. until emergence. They were then divided into four groups, of which two were kept subsequently at 25° C. and two at 15° C. Two groups, one at each temperature, were kept in vials in a saturated atmosphere; they are referred to as 15 W and 25 W.

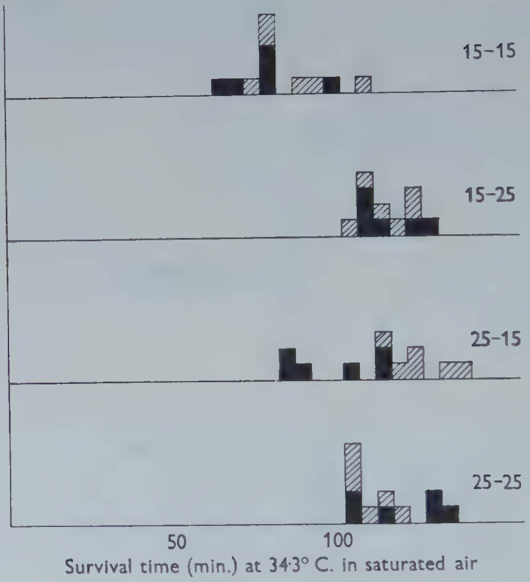

Fig. 3. Survival time in saturated air of flies previously kept at different temperatures. Symbols as in Fig. 2.

The other two groups were kept in food vials closed by cotton-wool stoppers in which particles of anhydrous calcium chloride were embedded. Drops of liquid appearing on the insides of these vials proved, when tasted, to consist of a concentrated salt solution. On the day of emergence, and on the next 3 days, the flies were transferred for 6 hr. to empty vials with anhydrous calcium chloride in the stopper; cobalt chloride filter papers remained bright blue in these vials. Rather surprisingly, all flies survived this rigorous treatment. These two groups are referred to as 15 D and 25 D. Three hours before testing, on the fifth day after emergence, the flies were transferred to fresh food vials with drops of tap water on the glass to afford them the opportunity of drinking.

All four groups were tested at 33·5° C. in dry air; the results are shown in Fig. 4. They show that physiological acclimatization, although it increases the capacity to resist desiccation rather than high temperature, is nevertheless caused by keeping flies at a high temperature, and not at a high saturation deficiency. Thus the 25 W group were greatly superior to the 15 W group, although both were kept in saturated air, whereas there was less difference between the 15 D and 15 W groups, and no significant difference between the 25 D and 25 W groups. Although unexpected, this finding makes sense ecologically, since in a species with a European distribution exposure to high temperatures will often be associated with the risk of desiccation.

Experiments described below suggest that the slightly longer survival times of flies kept in dry conditions are to be explained by the fact that these flies drank to excess when given the opportunity immediately before being tested.

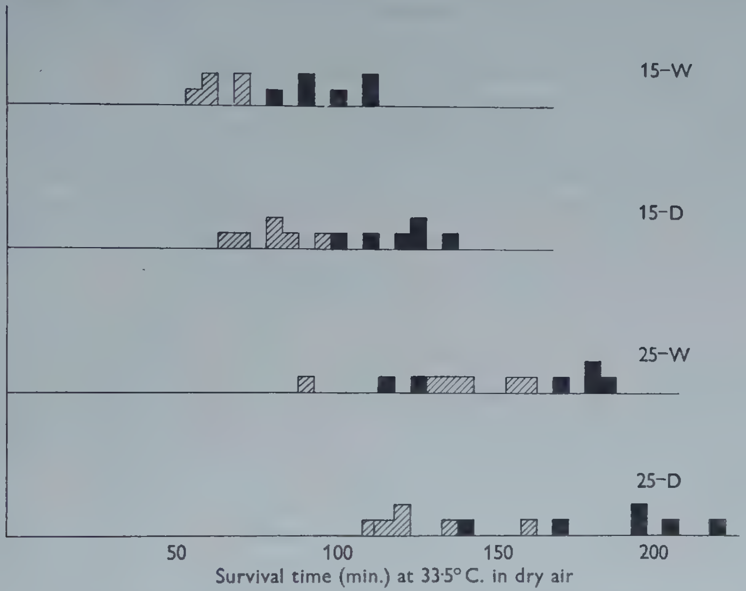

Fig. 4. Survival time in dry air of flies kept as adults in the following conditions: 15–W, 15° C. in saturated air; 15–D, 15° C. in dry air; 25–W, 25° C. in saturated air; 25–D, 25° C. in dry air. Symbols as in Fig. 2.

The results of all experiments on acclimatization to hot dry air are summarized in Table 1.

Table 1. *Mean survival times in minutes at 33·5° C. for flies with various environmental histories*

Temperature during adult life (° C.)	Temperature during pre-adult life (° C.)					
	15		20		25	
	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀
15	44	67	15D 79 15W 63	119 97	135	143
20			99	103		
25	112	155	25D 127 25W 135	188 159	165	204

REVERSIBILITY OF CHANGES OCCURRING AT HIGH TEMPERATURES

In dry air

By exposing individuals to high temperatures for approximately half the time required to kill them, and then retesting them after an intervening period at 20° C., it is possible to determine the reversibility of the changes which occur at high temperatures which would, if continued, result in death. Experiments of this kind have been performed only on females, which were kept at 20° C. until testing.

A number of females were exposed to 33·5° C. in dry air for 45 min.; allowing for the period of 10 min. during which the temperature was rising from room temperature to 33·5° C., and for the few minutes which elapsed before the flies could be removed from the test vials, this is equivalent to approximately 50 min. at 33·5° C., or to about half the mean survival time at that temperature.

After removal from the test vials the flies were kept for 3 hr. at 20° C. They were divided into three groups; group B was kept in an empty vial without food or water, group C in a vial with drops of tap water on the glass, and group D in a food vial with drops of tap water. A fourth group, E, was kept in food vials for 2 days at 20° C. The flies were then returned to the test vials, and their survival times measured at 33·5° C. in dry air, with the following results:

		Group	No. of females	Mean survival time at 33.5° C. in dry air (min.)
Control group		A	6	107·5 ± 4·0
Exposed for 50 min. at 33·5° C. in dry air followed by 3 hr. at 20° C.	No food or water	B	6	60·0 ± 7·5
	With tap water	C	5	149·0 ± 9·7
	With food and tap water	D	6	140·8 ± 5·7
Exposed for 50 min. at 33·5° C. in dry air, followed by 2 days at 20° C. in food vials		E	12	95·8 ± 3·5

These results suggest that death occurs as a result of the cumulative loss of water. Females of group B, which were not given water to drink between the first and second tests, survived for a total time of 50 + 60 min., closely resembling that of the control group. The two groups C and D, which were given water, survived in the second test for longer than the controls. Thus the changes which occur at 33·5° C. in dry air are fully reversed in 3 hr. at 20° C., provided that drinking water is available.

It is, however, a striking fact that the females which were given a drink survived for longer than the untreated controls, although, as shown by group E, the improvement is apparent only for a short time after the first exposure.

There are two possible reasons for this improvement after an initial exposure to 33·5° C. in dry air. First, it may be that females which were partly desiccated and then given water drank to excess, and so started the second test with a higher water content than did the controls. However, an alternative explanation was suggested by the behaviour of these flies. When first exposed to 33·5° C. they were extremely active, but after 50 min. became sluggish. When the temperature was then reduced to 20° C. they became quite passive, making no movements beyond those necessary to drink or to feed. In this state they could be tipped on to the bench and prodded with a paint brush without making any attempt to walk or fly away. This passivity continued for 3 hr., and when the temperature was again raised to 33·5° C. the flies were very inactive compared to the control group. Such inactivity, however, was no longer noticeable in group E after 2 days at 20° C. It is therefore possible that groups C and D survived longer because they were less active, and so lost less water in respiration.

These alternatives were investigated by exposing some females to desiccating conditions without raising the temperature, and others to a high temperature in saturated air, before recording their survival times at 33·5° C. in dry air. The results were as follows:

		Group	No. of females	Mean survival time at 33·5° C. in dry air (min.)
Control group		A′	10	102·5 ± 3·2
Flies kept at 20° C. in dry air for 18 hr., and then for 3 hr. at 20° C. with food and water		F	6	130·8 ± 4·9
Exposed for 50 min. at 34·3° C. in wet air, followed by 3 hr. at 20° C.	No food or water	G	6	93·3 ± 5·7
	With tap water	H	6	125·0 ± 5·2
	With food and tap water	I	5	125·0 ± 5·5

Group F were extremely active when raised to 33·5° C., as were the controls, whereas groups G, H and I were sluggish. Both group F, which had previously been desiccated, and groups H and I, which had been rendered sluggish by previous heating in saturated air, survived for longer than the control group A′, but none survived for as long as did groups C and D. This indicates that both the suggested causes for the longer survival of the latter groups were in fact operating. The short survival time of group G is, however, somewhat puzzling.

It is concluded that death at 33·5° C. in dry air is a consequence of desiccation, and that the changes involved are fully reversible; further, that the survival time can be slightly increased either by previous desiccation followed by an opportunity to drink, or by previous heating, which causes a great decline in activity, although neither of these factors seems sufficient to account for the extent of physiological acclimatization demonstrated in an earlier section.

In saturated air

A similar series of experiments was carried out on the changes occurring in saturated air, with the following results:

		Group	No. of females	Mean survival time at 34·3° C. in wet air (min.)
Control group		J	6	113·3 ± 4·4
Exposed for 50 min. at 34·3° C. in saturated air, followed by 3 hr. at 20° C.	No food or water	K	12	77·5 ± 5·5
	With tap water	L	12	87·9 ± 5·4
	With food and tap water	M	12	111·2 ± 2·7
Exposed for 50 min. at 34·3° C. in saturated air, followed by 20 min. at 20° C.		N	12	48·8 ± 3·3

The fact that group M survived for as long as did the controls suggests that the damage done during the previous heating in saturated air was effectively repaired in 3 hr. at 20° C., but not in 20 min. at that temperature (group N). Unlike groups C and D in the previous series, group M did not survive for longer than the controls, but this is not surprising, since earlier experiments had shown that physiological acclimatization does not greatly increase survival times in saturated air. Groups K and L suggest that complete recovery depends on the presence of food and possibly also of water.

DISCUSSION

The fact that flies which had previously been heated in dry air recovered completely if allowed to drink, but failed to do so in the absence of water, suggests that death in dry air may be due to the cumulative loss of water. However, although desiccation is a contributory cause of death, it cannot be the only factor, as is shown by two considerations.

First, the high value of $Q_{10} = 350$ for death in dry air is not consistent with the view that death occurs when a given quantity of water has been lost, either through the spiracles or by diffusion through the cuticle. The rate of loss through the spiracles would be approximately proportional to the product of the metabolic rate and the saturation deficiency, and would be unlikely to have a Q_{10} greater than about 5. Similarly, the activation energy for diffusion through the cuticle is not likely to be as great as is suggested by a Q_{10} of 350, as the following values show:

	Activation energy (cal./mol.)	
Mature *Tenebrio* pupae (up to 40° C.)	14,300	Holdgate & Seal (1956)
Tenebrio pupae abraded by mineral dust	12,300	Holdgate & Seal (1956)
8 species of aquatic insects	11,000–22,000	Holdgate (1956)
Various organic membranes	2,800–8,000	Barrer (1951)
Drosophila adults in dry air	109,000	

The value for *Drosophila*, calculated on the assumption that the rate of water loss is inversely proportional to the time of survival, is so high compared to the other values that it is improbable that diffusion of water through the cuticle is responsible for death in dry air.

Secondly, the similarity of the curves for wet and for dry air (Fig. 1) suggests that the causes of death may be similar in both cases. At the highest temperatures (35–36° C.) the survival times are in either case so short that little desiccation can occur in dry air, and consequently the dryness of the air does not alter the survival time. At lower temperatures, at which the flies can survive for several hours, appreciable desiccation may occur in dry air. If such desiccation increases the rate of the processes causing death, this would explain the shorter survival times in dry air. The processes of denaturation of enzymes and of proteins are known to have temperature coefficients approaching the value of $Q_{10} = 10{,}000$ ($E = 171{,}000$ cal./mol.) measured for death in saturated air. It is therefore possible that such processes are responsible for death both in dry and in wet air, and that the effect of desiccation is to increase the salt concentration in the body and so to speed up denaturation.

The nature of the changes in acclimatized flies which enable them better to withstand exposure to hot dry air is not known, although since acclimatization does not greatly increase survival times in saturated air, the changes presumably either reduce the rate of water loss, or enable flies to survive a greater percentage loss. A possible reason for a reduced rate of water loss in acclimatized flies is that such flies are less active, and so lose less water in respiration. It is known that some poikilotherms can compensate for a rise in temperature, so that their activity does not show a corresponding rise (Bullock, 1955), and this is in a sense true for *Drosophila*, whose activity at first increases at 33·5° C., but then falls to a low level. However, it has been shown that such a mechanism can account for only a small part of the increased survival time of acclimatized flies. Another possible factor is suggested by the work of Fraenkel & Hopf (1940), who showed that in two species of blowflies the temperature tolerance of larvae was increased if they were raised at a higher temperature, and that their phospholipids were more saturated, and thus had a higher melting point, than those from larvae raised at lower temperatures.

SUMMARY

1. The times for which adult *Drosophila subobscura* survived at high temperatures in dry and in saturated air were measured at different temperatures, over a range of survival times from 20 to 400 min. There is a linear relationship between the logarithm of the survival time and the temperature in both cases, the values of Q_{10} being approximately 350 in dry air and 10,000 in wet air.

2. Survival times in dry air were increased in individuals previously kept at a high temperature (25° C.). Two kinds of acclimatization can take place, a long-lasting 'developmental acclimatization' in individuals kept at 25° C. during pre-adult life, and a transitory 'physiological acclimatization' in adults kept at 25° C.

3. Survival times in saturated air were only slightly increased in individuals previously kept at 25° C.

4. Although physiological acclimatization increased the resistance of flies to desiccation rather than to high temperature as such, it is nevertheless a response to previous exposure to high temperatures, and not to high saturation deficiencies.

5. The reversibility of changes occurring at high temperatures was studied by exposing individuals for 50 min. to a temperature which would kill them in 100 min. and then retesting them after an intervening period at 20° C. Flies exposed to dry air recovered fully if they were allowed to drink; flies exposed to saturated air recovered in 3 hr. at 20° C., but complete recovery in this period required the presence of food and water.

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THE RESPIRATION OF *HELIX POMATIA*, A BALANCE SHEET

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INTRODUCTION

When one isolates a tissue from a living animal and carries out a physiological experiment upon it, one often assumes that the results obtained will be applicable to the tissues in the intact animal. This has proved a useful working hypothesis though there are few investigations into the validity of the assumption (Barcroft, 1908; Field, Belding & Martin, 1939; Martin & Furhmann, 1941, 1955).

The gastropods, and especially the snails, provide very suitable material for such an investigation, since their tissues are bathed in a blood sinus and do not have a specialized high-pressure blood supply canalized to each organ. With this in mind we have investigated both the respiration of the isolated organs of the snail and the respiration of the intact animal to see whether the sum of the parts added up to the respiration of the whole animal.

METHOD

Starved *Helix pomatia* were kept in a refrigerated cold room at 2–5° C. They remained in an inactive state throughout the period of captivity though none of them developed a calcareous epiphragm. The respiration measurements were carried out on thin slices of tissue placed in Warburg manometers. Unless otherwise stated the gas phase was air. Both the direct and the indirect methods were used (Umbreit, Burris & Stauffer, 1945). The manometer bath was kept at 28 ± 0·01° C., this being the temperature at which Baldwin (1938) carried out his studies on the metabolism of the snail liver. After each experiment the tissue was removed from the manometer, dried overnight and weighed.

The respiration of the whole snail was measured by the direct method, a simple container being fitted to the standard Warburg manometer. The bath was kept at 28° C. and the snails lived quite well at this temperature provided that the humidity was kept high. This temperature was well within the thermal limits of the animals; Hogben & Kirk (1944) state that snails will live for several days at 35° C. under humid conditions, whilst their upper thermal death temperature is 44° C. During the course of long-term experiments on the respiration of the intact animals it was periodically necessary to replenish the air in the chamber. This was followed by a period of re-equilibration.

RESULTS

(1) *Respiration of isolated tissues*

One of the first problems was the choice of a physiological solution to bathe the isolated tissue. Previous workers such as Baldwin had used a phosphate solution containing in each litre, 16·12 g. $Na_2HPO_4.12H_2O$., 0·69 g. $NaH_2PO_4.H_2O$, 0·14 g. NaCl, 0·443 g. KCl. It will be seen that this solution is mainly a phosphate solution bearing little relation to the constituents of normal snail blood (Lustig, Ernst & Reuss, 1937), though Baldwin took care to ensure that it was isotonic with snail blood and had the same pH and Na/K ratios. Rees (1953), in his investigation into the enzyme systems present in the liver of the snail, suspended his material in either 0·5 % KCl or 0·2 M-sucrose.

We decided to compare the respiratory activity of the tissues in Baldwin's solution and in a standard Ringer solution. For our Ringer solution we took Krebs–Ringer containing 0·15 M solutions of NaCl, KCl, KH_2PO_4, $MgSO_4.7H_2O$, and 0·11 M-$CaCl_2$ made up in the ratio of 100:2:1:1:3. It was buffered to pH 7·4 with phosphate buffer.

Table 1. *The respiratory rates of isolated tissues from* Helix pomatia *immersed in Baldwin's phosphate solution and Krebs'–Ringer*

Q_{O_2} = μl. oxygen/mg. dry weight/hr.

	Tissue	Readings	Q_{O_2} Baldwin	Q_{O_2} Krebs
1	Cerebral ganglia	20	4·00 ± 2·3	2·61 ± 1·7
2	Pedal ganglia	18	2·89 ± 1·6	2·53 ± 1·5
3	Liver	23	2·78 ± 0·42	1·39 ± 0·18
4	Gut buccal mass	24	1·37 ± 0·17	0·98 ± 0·11
5	Oesophagus	24	2·68 ± 0·25	1·63 ± 0·19
6	Mid-gut	24	2·56 ± 0·59	2·70 ± 0·40
7	Mantle	24	1·76 ± 0·29	2·31 ± 0·37
8	Kidney	27	2·24 ± 0·32	2·05 ± 0·18
9	Columella muscle	24	1·80 ± 0·34	1·17 ± 0·18
10	Female duct	24	1·03 ± 0·17	1·02 ± 0·20
11	Albuminous gland	22	1·20 ± 0·18	1·17 ± 0·18
12	Body wall	24	0·78 ± 0·08	0·94 ± 0·06
13	Dart sac	22	0·66 ± 0·07	0·59 ± 0·05
14	Foot: Fore	17	0·81 ± 0·11	0·67 ± 0·07
15	Middle	12	0·67 ± 0·08	0·70 ± 0·14
16	Rear	13	0·79 ± 0·06	0·86 ± 0·12

The results of these experiments are shown in Table 1. The rates of respiration are expressed in terms of Q_{O_2} (μl. oxygen/mg. dry weight/hr.). The tissues are arranged in order of oxygen consumption with the nervous system being the most active, then the liver, gut, mantle, kidney, followed by sundry organs with the foot having the lowest rate of respiration.

The values of Q_{O_2} showed a certain amount of variation as indicated by the size of the standard error. Much of this was due to variation between the tissues of different individuals and not due to the method of study. Thus when the liver from one animal was cut into slices and placed in eight manometers, the standard error of the series came to 0·06, whilst the standard error on the readings on liver slices taken from twelve different animals came to 0·42.

The values we obtained for the liver ($Q_{O_2} = 2·78 \pm 0·42$) agree with those obtained by Baldwin ($Q_{O_2} = 2·93$).

Table 1 shows that some of the tissues had a higher Q_{O_2} in Baldwin's solution than in Krebs's solution. The difference was most marked in the liver which was 95% higher in Baldwin's than in Krebs's, whilst the cerebral ganglia was 54% greater in Baldwin's. On the other hand, the body wall showed a Q_{O_2} that was 20% higher in Krebs's solution than in Baldwin's. This indicated that there might be a difference between the respiration of the internal and the external organs in the two solutions, the internal ones having the higher rate in Baldwin's solution and the external ones the higher rate in Krebs's. The difference between the internal and external tissues in the two solutions was found to be significant using the 't' test. The main exceptions to this generalization were the anterior part of the foot which had a higher rate in Baldwin's solution, and the mid-gut which had a higher rate in Krebs's solution.

The tissues of the intact animal are subject to varying concentrations of carbon dioxide and HCO_3^-. We studied the effect of various concentrations of CO_2 and HCO_3^- by the indirect Warburg method. We also determined the effect of adding HCO_3^- to the Ringer solution. Both methods gave essentially similar results; they led to an increase in the rate of tissue respiration. Thus use of Krebs's bicarbonate Ringer increased the Q_{O_2} from its normal value for liver of 1·39 to a value of 4·87. The gas phase was 5% CO_2, 95% O_2.

(2) *Respiration of the intact snail*

Snails were taken from the cold room and placed in the manometers. After a period of equilibration a series of readings were taken. The snails were sometimes found to respire slowly at first, but after 5 or 6 hr. the respiration increased to a 'steady' state. In fact this was not a steady level because all the snails showed considerable short-term fluctuations in their respiratory activity.

Fig. 1 shows the record of the respiration of a snail over the period of 100 min. It will be seen that the snail showed periods of high activity followed by low activity. We could not correlate these changes with either the opening and closing of the pneumostome, which was visible in many cases through the walls of the glass vessel, nor with the general muscular activity of the animal.

A series of more than one hundred readings, each of 15 min. duration, were taken over a period of 3 days, the snail remaining at 28° C. throughout this time. From these the value of the average Q_{O_2} for each snail was calculated. After the respiration had been measured, each snail was dissected and the organ systems removed, dried and weighed. A balance sheet was drawn up for each animal as shown in Table 2. The first column shows the organs in order of body weight. The body wall makes up 24% of the weight, the foot 20%, the liver 17% and the mantle collar 9%. The dry weight was approximately one-quarter of the wet weight.

Assuming that each of the tissues had been respiring at its average isolated rate whilst it was still in the snail, the weight of each tissue was multiplied by its average Q_{O_2}. These values were then added together and equal the average oxygen consumption of all the tissues of the snail in the isolated state, i.e. the summated tissue consumption.

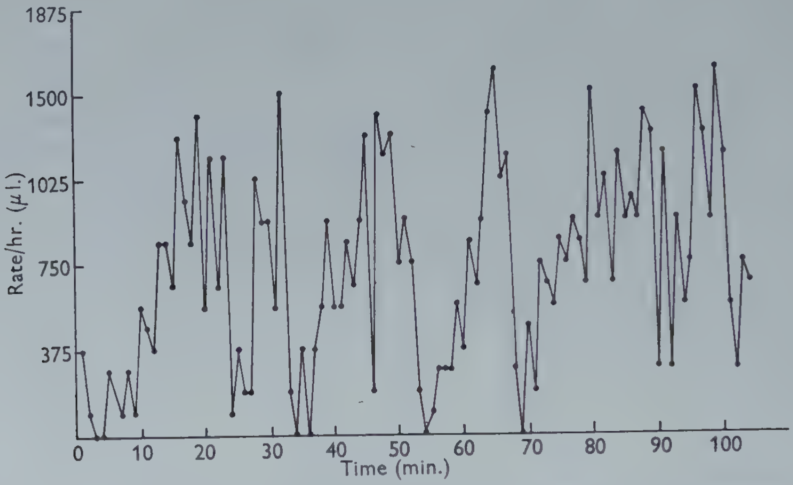

Fig. 1. The respiration of *Helix pomatia*. The rate of respiration is not constant but shows considerable fluctuations.

Table 2. *Balance sheet of tissue respiration in a single snail. For details see the text*

Tissue	Dry wt. (mg.)	Wt. × av. Q_{O_2} (Baldwin)	Wt. × av. Q_{O_2} (Krebs)
Body wall	339·0	264·0	319·0
Liver	233·0	648·0	324·0
Collar	128·6	103·0	159·0
Foot: Fore	102·1	82·2	68·1
Mid	101·0	68·7	71·4
Hind	87·6	69·5	75·4
Kidney	70·5	157·5	144·0
Female duct	68·9	71·0	70·5
Mantle	59·5	105·0	138·0
Oesophagus	39·2	101·0	64·0
Mid-gut	38·7	99·0	104·5
Buccal mass	31·8	43·8	31·2
Columella muscle	23·7	27·7	27·9
Dart sac	23·5	15·5	14·0
Albumen gland	18·5	23·9	17·0
Pedal ganglia	1·4	3·5	4·05
Cerebral ganglia	1·0	4·0	2·61
Total		1886 ± 287	1634 ± 108

The range of summated tissue consumption is indicated by the standard error for the summated average Q_{O_2} which is given by the following formula:

$$\text{S.E.} = \sqrt{\{(\text{Wt.}_{\text{liver}} \times \text{Av. rate}_{\text{liver}} \times \text{S.E.}_{\text{liver}})^2 + (\text{Wt.}_{\text{kidney}} \times \text{Av. rate}_{\text{kidney}} \times \text{S.E.}_{\text{kidney}})^2 + \ldots\text{etc.}\}}$$

Table 3 is a summary of the balance sheet of twelve animals. The values given under the headings Q_{O_2} Krebs and Q_{O_2} Baldwin are the summated average rates of respiration of the tissues of the snails in the respective solutions.

Table 3. *Comparison between summated tissue respiration and the respiration of the intact animal. For details see the text*

Animal	Summated tissue respiration		Whole animal Q_{O_2}	$\frac{\text{Whole animal}}{\text{Summated tissue}} \times 100$	
	Q_{O_2} Krebs	Q_{O_2} Baldwin		Krebs	Baldwin
1	1698 ± 91·8	1868 ± 223	1728 (397–1950)	102	93
2	2131 ± 108	2382 ± 278	2330 (45–2770)	109	98
3	2346 ± 139	2642 ± 358	1414 (890–2080)	60	53
4	1634 ± 108	1886 ± 287	1018 (520–1520)	62	54
5	2001 ± 110	2278 ± 319	2142 (78–3070)	108	95
6	1670 ± 106	2186 ± 304	1470 (635–1770)	88	67
7	1929 ± 123	2343 ± 423	1301 (640–2240)	67	55
8	1656 ± 102	1971 ± 294	700 (390–1160)	42	35
9	1563 ± 98	1827 ± 320	925 (400–2400)	59	51
10	1388 ± 89	1687 ± 292	1090 (780–1470)	79	64
11	2127 ± 117	2527 ± 381	1062 (170–2340)	50	42
12	2091 ± 124	2395 ± 356	844 (160–2000)	41	35

The Q_{O_2} of the intact animal is the average of at least one hundred readings taken throughout 3 days. The range—given by the side of each heading—indicates the extremes at which the animal respired, but they are not the standard error of the readings nor does the average Q_{O_2} of the intact animal necessarily fall in the middle of this range. They indicate that the snail can have a very high or a very low respiratory rate. This is also demonstrated by Fig. 2 which is a histogram of the frequency at which the different rates of respiration occurred in snail 7. It will be seen that though there is quite a broad range of readings, the average is considerably below the values marked for the summated tissue respiration in either of the two solutions.

Table 3 and Fig. 2 show that the highest rate of respiration of the intact animal never significantly exceeds the rate at which the tissues could respire, and in only three out of the twelve cases cited (nos. 1, 2, and 5) is there fairly close agreement between the average rate of respiration of the intact animal and that of the isolated tissues. In the other nine cases the animals respired at 40–70% the rate of the isolated tissues. The difference is most marked in the Baldwin solution. Since the average rate of respiration of the whole animal is most frequently lower than that of the isolated tissues, and since the animal respiring at its maximum rate *can reach* the rate of respiration of its isolated tissues, there might well be some type of control of respiration in the intact animal bringing its normal range of respiration to about 60% of the possible maximum rate.

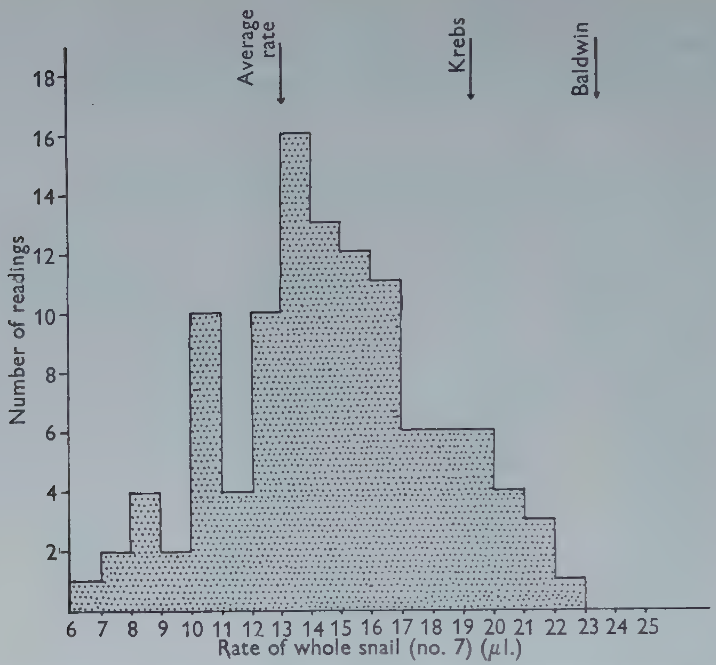

Fig. 2. Histogram of the frequency with which a snail respired at different rates. The average rate is marked for comparison with the rates at which the tissues respired in Krebs's and Baldwin's solutions.

DISCUSSION

The isolated organs of the snail each respire at a characteristic rate with the brain having the highest rate, then the liver, gut, mantle, kidney, etc. It is not unusual for the nervous system to have the highest repiratory rate in an intact animal. Thus McIlwaine (1955) pointed out that though the human brain is only 2·5% of the body weight it removes 25% of the oxygen taken in by the lungs. In most measurements on isolated mammalian tissues, however, the brain has a relatively low Q_{O_2}. Field *et al.* (1939) found the isolated organ hierarchy in the rat to be kidney, brain, liver, heart, etc. Krebs (1950), again working on the rat, found the order to be kidney, brain, liver, spleen. In both cases the brain respired at a lower rate than the kidney. McIlwaine (1955) has shown that the mammalian brain *in vitro* has a respiratory rate only 45% of the *in vivo* brain. If, however, the *in vitro* brain is stimulated electrically, the rate of respiration increases to 80% of its *in vivo* value. Such stimulation would bring the brain to a higher rate than the kidney. It is interesting to note that the isolated mammalian cortex show no electrical activity (Burns, 1951), whilst the isolated slug and snail brains show continuous electrical activity for up to 24 hr. after isolation (Hughes & Kerkut, 1956).

The relationship between tissue respiration and the basic metabolic rate has so far been analysed only for mammalian tissue. Field *et al.* (1939) carried out a respiratory balance sheet for the rat. They found that the isolated tissues respired at a lower rate than the whole animal. Thus the respiration of the whole animal was

151% of the summated tissue respiration. They suggested that if an allowance is deducted for minimal functional activity of the muscles *in vivo*, then the value for the whole animal would be some 112% the value of the isolated tissues. Martin & Furhmann (1941, 1955), working on the dog and mouse, found that the respiration of the whole animal gave a value of 112–103% of the summated tissue respiration in the dog, and 139% in the mouse.

Both these investigations differ from ours in that the isolated tissues are respiring at a slower rate than the whole animal. We, on the other hand, find that intact snails normally respire at about 60% of the tissue rate. This difference between the mammalian and molluscan situation may be explicable in terms of the blood supply to the intact tissues. In the mammals there is a well-organized blood supply carrying oxygen to within a short distance of all the cells and this is disrupted on isolation. In the snail, however, the tissues are for the most part bathed in a haemocoel and thus the isolated snail tissues are in a more normal respiratory situation than are isolated mammalian tissues.

This is supported by the fact that mammalian tissues respire better in a gas phase of pure oxygen whilst isolated snail tissues are unaffected by an increase in oxygen concentration.

Another difference between the behaviour of isolated mammalian and molluscan tissue is seen in the way in which the rate of respiration alters after the isolation of the tissue. In the mollusc the rate remains constant for the first 4 hr. after isolation. In mammals the rate falls off very markedly. Field *et al.* obtained their rates of tissue respiration at the time of isolation by extrapolation of a simple sloping curve. Lundsgaard (1950) has shown that the rate of respiration of isolated perfused mammalian tissue falls rapidly over the first $\frac{3}{4}$ hr. and then drops slowly. Field *et al.* made their first measurements about $\frac{3}{4}$ hr. after the tissue had been isolated, so in fact they extrapolated only over the gentle curve. This would then give them a low rate of tissue respiration and so explain why the respiration of the whole animals appears as 151% of the summated tissue respiration. Martin & Furhmann (1955) state: 'occasional high values of Q_{O_2} obtained for the first fifteen minutes of the experiment were not heavily weighted in plotting the results.' In one case they found that the basal metabolic rate came to 95% of the summated tissue respiration. One might expect that isolated tissue under ideal conditions should respire at a higher rate than they do in the intact animal, since this would provide a large safety factor in the working of the animal. The maximum summated tissue rate would then indicate the work potential of the animal.

In our experiments the intact snails showed a range of respiratory activity, but the maximum rate at which the animal respired never exceeded the rate at which we calculated its tissues could respire. In fact, we found that the animal was normally using only 60–70% of its maximum rate. This would indicate that the animal might have some means of controlling its tissue respiration. Barron (1943) has suggested that there may be some specific inhibitory control of intracellular reactions. He bases this view on the fact that the rate of a reaction of an isolated enzyme system is many million times faster than that of the same reaction in the tissues and cells themselves. It is, however, difficult to tell the extent to which the rate of cellular reactions is due to inhibited, and thus controlled, systems and the extent to which the rate is due to enzyme systems diluted down with 'inert' materials.

There is some evidence for hormonal control of tissue respiration in other animals. Lardy & Maley (1954) found that thyroxin affected the rate of respiration of isolated mammalian mitochondria. In experiments where phosphorylation was controlled they found that the rate of respiration was dependent upon the thyroxin concentration, being increased by low concentrations and decreased by high concentrations.

Samuels (1956), working on the cockroach *Leucophaea*, compared the respiratory rates of isolated normal thoracic muscles and those from animals that had had their corpora allata removed 3 months previously. He found that the muscle from the allectomized animals had a 20% higher rate of respiration than had normal muscles. This would indicate that the respiration in the normal thoracic muscles is inhibited in some way by the action of the corpora allata.

On the other hand, Thomsen (1949) found that removal of the corpus allatum from *Calliphora* led to a drop in the oxygen consumption of the whole fly by 24%. Implantation of extra corpora allata led to an increase of 19% in the oxygen consumption. Samuels suggests that the difference between these results may be due to the fact that he was working on isolated tissues whilst Thomsen was working on whole animals, and that removal of the corpora allata from intact animals might lead to a lowering in the blood sugar and hence a lowering in the substrate for muscle respiration.

There is at present little or no evidence of hormone action in gastropods though there are histological studies indicating the presence of neurosecretory cells in the ganglia of *Buccinum*, *Murex*, *Nucella*, *Archidoris* and *Aplysia* (Scharrer & Scharrer, 1954; Gabe, 1953). We are carrying out a series of experiments into the action of various substances on tissue respiration in molluscs and are also determining the effects of removing various parts of the snail on the respiration of the whole animal.

SUMMARY

1. The respiratory rates of tissue slices from the various organs of *Helix pomatia* have been determined. The internal organs such as the brain and liver show a higher Q_{O_2} when immersed in Baldwin's phosphate solution. The external organs such as the mantle and collar show a higher Q_{O_2} when immersed in Krebs–Ringer solution.

2. The brain has the highest Q_{O_2}; it is followed in order by the liver, gut, mantle, kidney, columella muscle, female duct, albuminous gland, body wall, dart sac and foot.

3. The rate of respiration of a series of intact snails has been determined. The organs from each of these snails were removed, dried and weighed. From the respiration rates determined in the early part of the paper, a balance sheet was drawn up for each snail. The summated tissue respiration is compared with the respiration of the intact animal.

4. In three out of the twelve cases cited there was good agreement between the summated tissue respiration and the respiration of the whole animal. In the nine other cases the respiration of the whole animal was 40–70% below the summated tissue respiration. This would indicate the possibility that the snail has some means of controlling the rate at which the tissues respire in the intact animal.

We should like to thank Dr K. A. Munday and Prof. W. T. Williams for valuable criticisms and comments.

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FURTHER OBSERVATIONS ON CO-ORDINATION AND THE DETERMINATION OF FREQUENCY IN THE PERISTOMIAL CILIA OF *STENTOR*

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INTRODUCTION

In an earlier paper (Sleigh, 1956) it was shown that the mechanical process involved in ciliary activity functions independently of the co-ordination process. The conduction of metachronal waves is independent of mechanical factors, and involves the successive excitation of cilia along the row.

Such a transmission process could function in one of two ways. It could be the result of a continuous neuroid conduction process along the length of the peristomial row of cilia, so that cilia are stimulated to contract as the impulses pass but do not themselves take any part in the transmission of the metachronal impulses; alternatively, it could be the result of a step-by-step process passing from one cilium to the next, so that the excitation of one cilium would result in the production of an excitatory impulse responsible for the stimulation of the next cilium.

If the conduction process is of the first type, the neuroid transmission is very slow (about 600μ/sec. at 20° C.; Sleigh, 1956) compared with that found in other conducting tissues. In the second type of transmission, however, there are two parts: the intraciliary excitation process, and the interciliary conduction process. Here the actual conduction process could easily be of a speed comparable with that in nerve and heart muscle, provided that the intraciliary excitation process occupies a large part of the total time of the conduction. There is some evidence for this; the wave conduction process in *Mytilus* lateral gill cilia, described by Gray (1930), is much slower than that in *Stentor*, but in *Mytilus* many more cilia are involved in the formation of each metachronal wave. Also, it was shown in the author's previous work on *Stentor* that the metachronal wave velocity was much increased by digitoxin; this drug lowers the threshold of excitability in heart muscle (Sollman, 1950), and might similarly affect the intraciliary excitation process.

The rate of beat of a cilium will normally be controlled by a series of excitatory impulses transmitted by one or other of the methods mentioned above. Individual isolated cilia, such as the abfrontal cilia of *Mytilus* studied by Gray (1930) or cilia of ciliate protozoans isolated with a small mass of protoplasm by Verworn (1889), show a spontaneous rhythmic beat. Thus the conducted excitation cannot be the only method of evoking a response in a single cilium.

MATERIALS AND METHODS

The *Stentor* used in these experiments were from the same stock as those used earlier (Sleigh, 1956), and were cultured by the same method. The movements and metachronal characteristics of the peristomial cilia of the animal were briefly described in that paper. Measurements of the frequency of beat were made by means of the rotating mirror stroboscope, and the wavelength was measured from photographs; the product of these two measurements gives the velocity of metachronal wave transmission. The temperature was kept approximately constant during the measurements by the method previously described, and was between 18 and 20° C. in all of the experiments.

In the microdissection experiments, cuts in the peristome edge of *Stentor* were made with glass microneedles made for this purpose in a de Fonbrune microforge. The animal was placed in a hanging drop on a glass slide which was raised on blocks on the stage of a microscope during the operations. When the animal had completely expanded, the edge of the peristome was cut in an appropriate place by compressing the animal against the glass slide with the glass needle controlled by a de Fonbrune micromanipulator.

EXPERIMENTAL

A. *The effect of a cut across the peristomial row of cilia*

Several authors have claimed that a cut across a row of cilia resulted in a disorganization of the co-ordination of the row. Experiments have been carried out on ctenophores by Verworn (1889) and Parker (1905), and on ciliate protozoans by Verworn (1889) and Taylor (1920). The precise nature of the 'disorganization' is not given, though Verworn describes differences in rhythm, and his figures show different metachronal wavelengths on the two sides of the cut. On this evidence a cut may result in a change in either the frequency, or the wave velocity, or both.

The arrangement of the peristomial cilia of *Stentor* is shown in Fig. 1. Waves of activity pass along the length of the row from one end to the other, starting from the gullet region. The frequency of beat is normally the same all along the row.

In a typical experiment, several readings of the frequency and of the wave velocity of the peristomial cilia were first taken. The peristome edge was then cut across in one or two places, and the frequency and wave velocity of the various regions were again measured as quickly as possible. Further measurements were taken at intervals until the cut had healed. The cuts usually resulted in a disturbance of the normal rhythm of the system, so that the cilia on the two sides of the cut beat with a different frequency. Correlated with this difference in frequency was a difference in wavelength.

Calculation of the wave velocity indicated that it was approximately the same on the two sides of the cut in each experiment. The difference in wave velocity between the distal region and the proximal region varied between $-3{\cdot}7$ and $+4{\cdot}3\,\%$, with a mean from seventeen experiments of $-0{\cdot}36\,\%$ and a standard error of the mean of 0·57 %. This is close to the normal variation in measurements of wave velocity, and the mean difference is not significantly different from zero. Although the cut interrupted the wave conduction process, this interruption did not change the wave velocity in any part of the peristome.

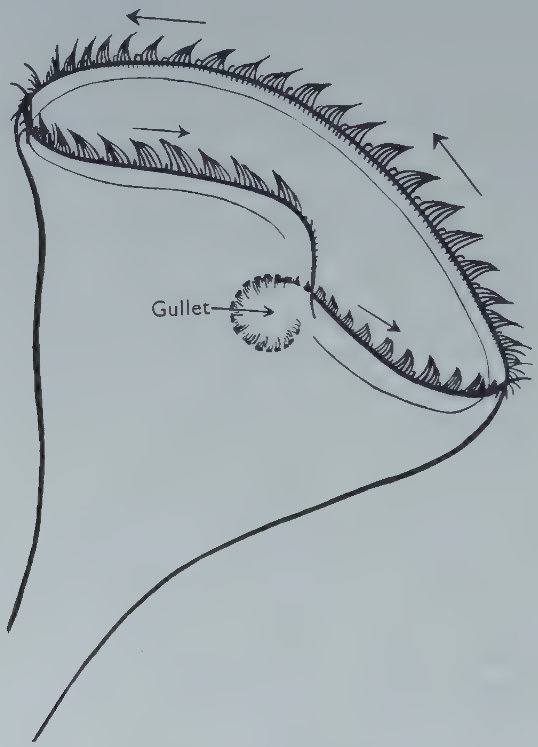

Fig. 1. The peristome of *Stentor* showing the arrangement of the cilia and the direction of metachronal wave transmission.

The frequency differences were much larger; the maximum recorded was 19·1 %, while the mean difference in frequency on the two sides of the cut was 8·4 %. The differences within individual experiments were statistically significant in nearly every case, but the differences were very variable. When several cuts were made in the row each region showed a different frequency; examples of results from five experiments of this type are shown in Table 1.

Within each isolated region all cilia beat with the same frequency, and it was usually found that the frequency in the proximal region was the same as the frequency along the whole row before the operation. Immediately distal to the cut, new waves of activity of a different frequency were propagated towards the distal end of the row. The cilia on the distal side of the cut beat more slowly than those on the proximal side in twenty-two cases out of twenty-nine, while in the other seven cases the distal frequency was greater than that proximal to the cut. The magnitude of the difference in frequency could not be correlated with the position or size of the cut. The cut healed in 15 min. to an hour or so, depending on the size of the cut. On healing, the distal frequency reverted to the original value and the frequency was the same throughout the row. This change in frequency usually took place in a single jump, like that which occurred when the cut was made, but occasionally it was more gradual as physical continuity was re-established.

Table 1. *The frequencies (beats/sec.) measured in sections of the peristome row which had been isolated by cuts*

Before operation	After operation			
	Proximal section	Distal isolated sections		
		1st	2nd	3rd
29·2	29·4	27·6	26·3	24·7
29·3	29·2	27·4	29·9	—
30·2	30·5	27·3	26·2	27·0
29·8	29·0	26·7	28·7	—
35·1	35·4	38·6	35·3	—

These experiments indicate that the existence of a common frequency over the whole row depends on physical continuity for the whole length of the row. This physical continuity can be broken by a cut in the row, but it has also been observed to be broken by a sharp bend in the peristome edge, such as might be caused by the contraction of one or two myonemes in the *Stentor* body wall, or by the peristome edge being caught up by an object in the environment. Such a discontinuity invariably resulted in a decrease in the frequency of the distal part. On other rare occasions the uniform frequency along the row may break down without any obvious cause. In all these cases there is apparently some failure of the transmission mechanism, a failure which separates the cilia distal to the 'break' from the proximal cilia. A new frequency is set up in the isolated region, which is the same for its whole length and which seems to be determined quite arbitrarily, because it bears no particular relation to the frequency with which the cilia were beating previously.

The observation that the conduction process can stop and start at any place tends to suggest that the transmission is not by a continuous neuroid conduction of the first type above, but by the second method involving a series of alternate excitation and conduction stages, in which the cilia are involved in the transmission process.

B. *The wavelength and wave velocity in different parts of the peristomial row of cilia*

The cilia in the gullet region appear to have a shorter metachronal wavelength than those in the main part of the row, as shown in Fig. 1. Since the frequency is the same throughout, this means that the metachronal wave velocity in the gullet region is less than that farther along the row.

Measurements of the metachronal wavelength of peristomial cilia taken as close as possible to the gullet were compared with measurements made in other parts of the row. In every case it was found that the wavelength was shorter in the gullet region than it was distally, and that the first wave was the shortest. The wavelength increased with each successive wave until it reached a maximum and thereafter remained the same throughout the distal part of the row. Results from seven sets of these observations are given in Table 2.

Table 2. *The wavelength and wave velocity in the gullet region and in the more distal part of the peristomial row*

Wavelength (μ)		Frequency (beats/sec.)	Wave velocity (μ/sec.)	
Gullet region	Distal		Gullet region	Distal
12·1, 13·3, 15·0, 16·7	23·0	31·5	381, 418, 472, 526	724
11·7, 13·8, 15·8, 17·5	23·4	26·5	310, 366, 418, 464	620
15·4, 16·7, 18·3	24·2	25·6	394, 427, 468	619
13·8, 14·6, 17·1	23·8	27·8	383, 406, 475	662
13·8, 16·3, 18·8, 20·8	23·5	28·2	389, 459, 530, 586	663
14·6, 16·3, 17·9	27·5	24·8	362, 404, 444	682
13·8, 17·1, 18·8	24·8	27·1	374, 462, 509	672

This variation in the rate of conduction will not fit in with either of the transmission mechanisms outlined above. However, a further investigation of the cilia of the gullet region revealed the reason for this variation in wavelength and wave velocity. The cilia in this region are smaller and closer together than those in the distal region; as they become larger and farther apart the wavelength increases. Counts of the number of cilia concerned in the formation of each metachronal wave showed that the number of cilia per wave was the same whatever the wavelength, even though the wavelength in the distal region was about twice that of the first proximal wave measured. Seven sets of observations illustrating this are given in Table 3.

Table 3. *The number of cilia in each wave in the gullet region and in the more distal part of the peristomial row*

Exp. no.	Gullet region					Distal region				
	No. of waves	Total no. of cilia	Cilia/wave	Average wavelength (μ)	Mean inter-ciliary distance (μ)	No. of waves	Total no. of cilia	Cilia/wave	Average wavelength (μ)	Mean int ciliary distanc (μ)
1	3	18	6	15·8	2·63	4	24	6	22·1	3·68
2	2	12	6	15·8	2·63	3	18	6	24·2	4·03
	3	18	6	13·3	2·22					
3	4	24	6	13·5	2·25	3	18	6	23·0	3·83
4	2	12	6	15·4	2·57	4	24	6	24·6	4·10
	4	24	6	17·7	2·95					
5	4	22	5·5	17·4	3·17	9	52	5·8	23·5	4·05
	4	24	6	16·9	2·82					
6	3	18	6	16·6	2·77	2	12	6	24·8	4·13
7	3	18	6	16·6	2·77	4	24	6	27·5	4·58
	3	18	6	16·3	2·72					

These data can be compared with those from other experiments. The average number of cilia per wave in forty-two normal animals was 6·4 and the average frequency in 361 normal animals at 18·5° C. was 27·7 beats/sec., so that the average number of cilia stimulated per second by a single metachronal impulse is about 178 and the average phase difference between successive cilia is about 5·6 msec. The average wavelength in 303 normal animals was 23·8μ, and thus the average interciliary distance was 3·9μ. The figures for the distal region shown in Table 3 agree closely with the average figures, but the corresponding values for the wavelength and the interciliary distance in the gullet region are much smaller than the average, although the number of cilia per wave is approximately the same.

Since the number of cilia in every wave is the same, the number of cilia stimulated per second by a single metachronal impulse must be the same all along the row, and the phase difference between adjacent cilia will be constant (about 5·6 msec.). The wave velocity thus depends on the number of cilia involved in the transmission, and not on the linear distance travelled by the metachronal wave. Evidence is thus strongly in favour of a transmission mechanism in which the cilia themselves take part.

DISCUSSION

I. *The metachronal transmission process*

Metachronal co-ordination has been explained in the past both as a mechanical process and as a neuroid transmission process, and evidence has been put forward in favour of both theories. Kraft (1890) found that a stimulus causing increased activity in a ciliated epithelium was transmitted across a region of quiescent cilia without any mechanical activity in the quiescent region. At about the same time Verworn (1891) found that there was no wave conduction past a ctenophore swimming plate that was not allowed to beat, but that waves were conducted normally if it was allowed to beat without touching any other plate. However, Parker (1905), working on *Mnemiopsis*, found that conduction was not interrupted when a plate was held still, nor when a plate was removed from the row. This latter experiment resembles experiments of Verworn (1889), who noted that ctenophore swimming plates on the two sides of a cut showed different rhythms and continued to beat actively. All these experiments indicate that metachronal co-ordination is by a neuroid transmission rather than by a mechanical process.

Gray (1930) thought that a neuroid transmission mechanism was unlikely for the following reasons: (1) metachronal waves can arise or be suppressed at any point along an epithelium, and cannot be traced to a centre from which the exciting stimulus can be shown to arise; (2) isolated ciliated cells are nearly always active; (3) the rate of metachronal transmission is very much slower than that of any known nervous impulse. He therefore suggested a mechanical triggering mechanism, in which one cilium excites the next when it reaches a certain stage in its own contraction cycle. This is supported by the present work, because the phase difference between adjacent cilia is constant throughout the peristomial row in *Stentor*, although the interciliary distance is variable. Previous work on *Stentor*

(Sleigh, 1956) showed, however, that the phase difference was readily changed in a viscous medium or with magnesium ions without any change in the rate of wave transmission, while digitoxin reduced the phase difference and increased the rate of wave transmission.

It seems likely therefore that some type of excitatory transmission process is involved, in spite of the reasons which led Gray to discard such a theory. This type of process would be comparable with the conduction of the contraction wave in heart muscle. Both processes represent an excitation mechanism responsible for eliciting activity in contractile structures by means of transmitted impulses.

The evidence accumulated concerning ciliary co-ordination can be interpreted in two ways, according to the two theories of transmission mentioned in the introduction: (1) conduction is a continuous neuroid process, the velocity of which is slow in the gullet region and increases to a maximum in the distal part of the row; (2) conduction is a step-by-step process involving successive excitation from cilium to cilium along the row. If the second theory is accepted, then since the phase difference between successive cilia has been found to be independent of wide variations in interciliary spacing, it follows that any time involved in interciliary conduction must be short compared with that required for the excitation of each cilium. The results obtained by Kraft and Parker fit the first type of process, and are less easily explained in the second theory, while the work of Verworn and the present work on *Stentor* indicate that the second type of process is involved. Gray's objections to a neuroid transmission process apply to the first theory, but are easily explained by the second, so that it seems as if the step-by-step transmission is the more likely hypothesis.

Both excitation and conduction are involved in such a step-by-step process, and the transmission will have properties belonging to both types of process. Changes in the conduction process would not be obvious since it occupies a much smaller time than the excitation process, from which it is not at present experimentally separable. The intraciliary process may be thought of as the building up of an 'excitatory state', under the action of an impulse from the previous cilium; discharge of this excitatory state would be responsible for triggering off (i) the contraction of the cilium, and (ii) the next conduction phase. Wave velocity will be most easily affected by agents which affect the reactions responsible for this build-up of excitation, e.g. digitoxin, which lowers the threshold for excitability in heart muscle, and may have a similar action here.

II. *The determination of frequency*

Since the contraction and transmission processes are to a large extent independent, the method by which the frequency of beat is determined still remains to be considered. Isolated cilia often show contractility; the beat of an abfrontal cilium in *Mytilus* is quite unrelated to that of any other cilia on the gill, and frequently exhibits long interkinetic periods. If we assume that the excitation of this cilium is intrinsic, we can visualize a fall in the threshold of excitability until the process becomes self-triggering. In *Stentor*, however, the constancy of the frequency along the row clearly indicates that the triggering is extrinsic—it may be assumed that this occurs at a somewhat higher threshold of excitability than that which produces spontaneous excitation, which will therefore not take place; the cilia will beat at a higher frequency than that of their spontaneous beat.

It is still true, however, that the frequency in *Stentor* can be significantly reduced by increasing the mechanical resistance (viscosity) of the medium; this observation appears incompatible with the hypothetical mechanism of excitation outlined above. It becomes necessary to assume that the excitation of a new contraction is delayed until the cilium has completed its contraction against whatever external resistance may be applied. However, the pacemaker of the whole system in *Stentor* appears to be situated in the gullet region and it is reasonable to suppose that it is one of the cilia in that region. If so, the effect of a viscous medium on the pacemaker and on the paced cilia will be similar, and the control of the distal cilia by the proximal ones may still be governed by an excitation mechanism. In this way it is possible to understand why a change in viscosity affects the frequency without altering the rate of conduction, whilst the addition of digitoxin, which changes the wave velocity by altering the excitability of the individual cilia, invariably affects the frequency as well, presumably by its action on the cycle of excitability of the pacemaker.

These ideas may be compared with the conduction system in the vertebrate heart, whose properties were discussed by Davies & Francis (1946). The different chambers of the frog heart all have their own intrinsic rhythms, decreasing in the order: sinus, atria, ventricle, bulbus cordis. Normally, the four parts all beat with an identical rhythm which originates in the sinus musculature and is conducted throughout the heart. The pacemaker system is very like that postulated for cilia, and it is interesting to note that cilia can beat at different frequencies under the control of different pacemakers, as do the chambers of the heart when isolated. Thus cuts across a row of cilia result in the setting up of new frequencies in the isolated regions; these new frequencies depend on a new rate of stimulation of the cilia initiated by a cilium at the beginning of the region. The new frequency was usually slower, but occasionally faster, than the original frequency, and it is possible that the fast controlling rate in the cilium next to the cut resulted from a local injury, since the observed discontinuities of rhythm in the absence of physical damage always resulted in a decrease in frequency at the discontinuity. Thus it seems likely that the intrinsic stimulation rate decreases from the gullet to the distal end of the row in the same way as the intrinsic rhythm of the various chambers of the heart decreases from sinus to bulbus cordis. This is borne out by the observation that the disturbance in the gullet region when the *Stentor* swallows food results in a temporary drop in the frequency, presumably because a cilium further down the row is acting as a temporary pacemaker.

The combined action of the pacemaker and transmission mechanisms is illustrated in Fig. 2. This shows a pacemaker cilium whose frequency is imposed by transmitted impulses on two of the cilia which follow it. The frequency of the row of cilia depends on the rate of contraction and the rate of excitation of the pacemaker cilium, the slower factor being the limiting one, and on the ability of the paced cilia to beat as fast as the rate set by the pacemaker. The wave velocity depends on the rate of the interciliary conduction and on the time taken by the intraciliary excitation process in each participating cilium.

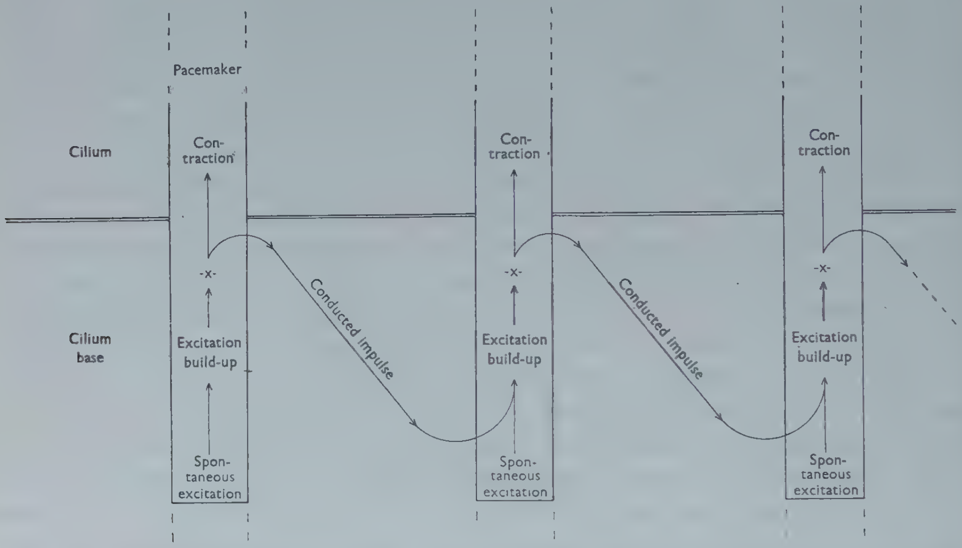

Fig. 2. A diagrammatic representation of the theory of metachronal co-ordination proposed in the text. The spontaneous build-up in the pacemaker cilium determines the frequency in that cilium, and the other cilia beat at constant intervals after this as a result of the conducted impulses.

Bradfield (1955) explains the ciliary contraction cycle as the result of a sequence of propagated contractions in the peripheral fibrils, involving the passage of waves of excitation in both directions around the cilium simultaneously. In either direction there will be five excitation stages concerned in the initiation of the propagated contractions. The average phase difference between successive cilia in *Stentor* is about 6 msec. Much of this time is taken up by the excitation process within the cilium, and it is not unreasonable to assume that each of the five fibrillar excitation stages takes about 1 msec. In the light of the theory that nervous tissues arose from ciliated epithelia, it is interesting to speculate on the similarities in timing and character between the excitation process outlined above and that at a nerve synapse.

SUMMARY

1. The work described is a continuation of the work on the peristomial cilia of *Stentor* previously reported (Sleigh, 1956). The same methods were used in making the observations.

2. A cut across the peristome row of cilia did not affect the wave velocity, but the frequency distal to the cut was usually altered. In twenty-two cases the distal frequency was decreased, and in seven cases it was increased.

3. Cilia in the gullet region are smaller and closer together than those in the main part of the row; these cilia show the same frequency as the other cilia, but a smaller wavelength and wave velocity. The number of cilia in every metachronal wave of the row is the same, as is the number of cilia stimulated in unit time by a single metachronal impulse in any part of the row.
4. The wave velocity is dependent on the number of cilia through which the conduction is passed. The transmission is thought to be the result of a rapid conduction process between the cilium bases and a slower build-up of an excitatory state in the cilium.
5. The frequency of a group of cilia is dependent upon the activity of a pacemaker at the beginning of the group; this pacemaker is probably a cilium with a suitable rate of beat. Frequency is limited by the rate of stimulation or the rate of contraction, whichever is the slower.
6. It is suggested that there are some five excitation stages in the excitation of each cilium, and that each stage takes about 1 msec.; this is comparable with the delay at a nerve synapse.
7. The results of these experiments and observations on ciliary activity indicate that the pacemaker and transmission properties of ciliated tissues and cardiac muscle are comparable in many respects.

It is a pleasure to thank Prof. J. E. Harris for his critical interest and continued guidance in this work, which was carried out during the tenure of a grant from the Department of Scientific and Industrial Research.

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STRUCTURE AND FUNCTION IN THE NEMATODES: INTERNAL PRESSURE AND CUTICULAR STRUCTURE IN *ASCARIS*

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No one who has made even a casual study of the Nematoda can fail to have been struck by the extraordinary similarity in form and organization between the very large number of species, genera, orders and families which constitute this class of the very diversiform phylum Aschelminthes. Though there are species which show some variation from the normal 'nematode facies', they are few in number and merely seem to emphasize still further the great uniformity of the group as a whole. The characteristic features of the nematodes are present in fresh-water, marine and parasitic forms; they are largely independent of size, of diet and of stage of development.

This uniformity can hardly be ascribed to simplicity of organization. Well below the level of complexity of the nematodes, the protozoa, sponges and coelenterates show a rich variety of adult forms and of larval stages within comparatively restricted taxonomic or ecological groupings. The platyhelminths, otherwise similar in their degree of organization so far as the presence of specialized tissues and organs are concerned, show a far greater diversity of form and structure; the elementary student may be forgiven at times for thinking that there is only one nematode but that the model comes in different sizes and with a great variety of life histories.

Among the factors which act as powerful determinants of organic form and structure, we owe to D'Arcy Thompson the idea that physical and perhaps more particularly mechanical factors are highly significant. Even in man-made structures this is equally true. A bridge, a ship, an aeroplane are recognizable at once because their design is based necessarily and largely on the mechanical forces which play such a predominant part in their economy; the decorative and even to some extent the functional variations which may be added to the structure are severely limited by the need to satisfy the rigorous requirements of mechanical strength and efficiency. Is it in such limitations that we may find the key to the underlying uniformity in the nematodes?

We believe that this is so. The popular name for nematodes—roundworms—indicates that their characteristic shape is that of an elongated cylinder, a shape which is often dictated by mechanical considerations. Internally, one of the most striking and invariable characteristics of the class is the possession of a body musculature composed entirely of longitudinal muscle fibres. Since the successful functioning of any system of muscles must provide for their contraction against a suitable antagonistic force, in the absence of circular musculature there must be an elastic return mechanism which could be provided by an internal hydrostatic pressure. The extent to which this system will be a determining factor in the organization of the nematode body will clearly depend on the magnitude and distribution of the forces involved.

The present paper, the first of a series in which the general biology of the nematode body is considered in mechanical terms, is concerned with the determination of the internal turgor pressure, its relationship to the structure of the cuticle and to the force exerted by the longitudinal musculature.

MATERIAL AND METHODS

The present studies have been made on adult female specimens of *Ascaris lumbricoides* obtained from freshly killed pigs from a Bristol slaughterhouse. The worms were brought to the laboratory in a limited volume of pig intestinal fluid contained in a previously warmed wide-mouthed vacuum flask. Experiments were carried out in the medium recommended by Hobson (1948)—30% sea water—in which the worms remain active for long periods. Not only do whole worms survive well in this artificial medium, but portions of worms such as those used in some of the additional experiments, reported below, survive and maintain their characteristic activity for several days. All experiments were, however, carried out as soon as possible after the arrival of a fresh batch of worms, usually within 6 hr. of obtaining the specimens.

Two methods of recording the turgor pressure have been employed—a direct method using a pressure gauge connected with a cannula inserted into the body cavity, and an indirect method based on the distortion of the body wall when external pressure is applied.

The essential features of any pressure gauge which is to be used for the direct recording of the internal body pressure are that it must be sensitive, rapid in response, and that it must require very little change in internal volume to bring about movements of the indicator. The last two requirements rule out the use of an ordinary manometer, such as that employed by Chapman & Newell (1947) in their work on *Arenicola*, but are admirably met by the 'spoon gauge' recorder, used by Picken (1936) in his studies on the turgor pressure of arthropods. The very small movement of the needle in the spoon gauge makes it necessary to use a low-power microscope to observe its deflexions, and this is a troublesome complication. Fortunately, we discovered in use in the Chemical Laboratory a simple variant of this type of gauge which served our purpose admirably. The recording element consists of a ribbon of very thin-walled glass tubing, less than 1 mm. in width, coiled into a cylindrical helix of twelve turns, 1 cm. in diameter. When pressure is applied internally the coil unwinds, and since the effect of the turns is additive, the angular deflexion at one end of the helix when pressure is applied to the other is considerable. These pressure gauges are normally filled with air, but it was a simple matter to fill them with boiled distilled water under vacuum and then to seal off the tip.

The open end was connected through one arm of a T-tube by a continuous water column to the source of pressure to be measured; all connexions were made with glass tubes jointed with cement or sealing wax to reduce changes in internal volume of the instrument to a minimum. It was found convenient to cement the base of a hypodermic syringe on to the pressure tip so that various types of cannula modified from ordinary hypodermic needles could readily be attached and replaced. The second arm of the T-tube led through a simple tap to a vertical water column providing a pressure head. This was used to flush out the cannula or to indicate if it was blocked, and also, when the opening of the cannula was sealed off, to calibrate the gauge.

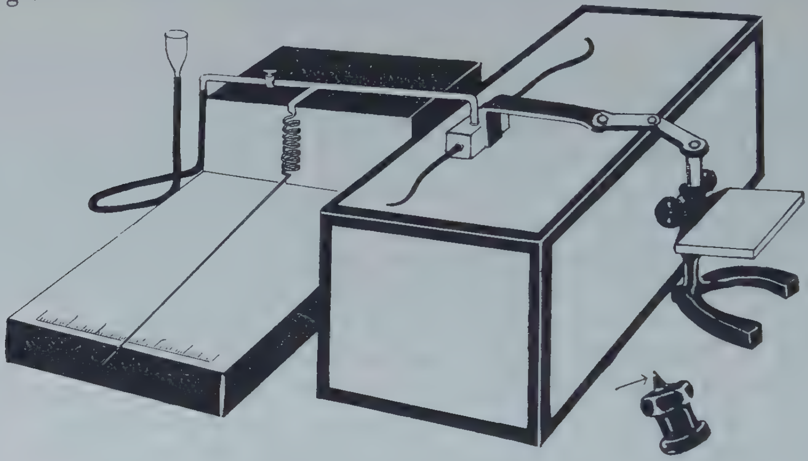

Fig. 1. The use of the glass helix pressure gauge. *Inset* (lower right), an enlarged view of the cannula. The arrow points to the lateral opening.

To the closed end of the helix was fixed a light black glass pointer, some 15 cm. in length, which moved over a millimetre scale. Calibration showed that the movement was linearly related to the pressure over a wide range, a deflexion of 10 mm. corresponding to a pressure of 55 mm. of mercury. The natural period of oscillation of the system was less than 0·5 sec. and the response was quite sufficiently rapid for the preparations used; reasonable shielding of the needle from stray air currents was all that was required.

Considerable trouble was encountered in finding a suitable form of cannula, which required to be sharp in order to penetrate the tough cuticle of the animal, but steeply tapered in order to seal the puncture successfully. Best results were obtained by soldering a small blob of metal around the base of a hypodermic needle which was then ground down to a length of 1–2 mm. The metal blob was then trimmed to a conical shape with an apical angle of about 45°. The hole in the tip was at the side, to reduce the chance of blocking during insertion, and was about 0·2 mm. in diameter.

In order to support the animal during the measurement it was threaded through a cylindrical hole, 6 mm. in diameter, in a block of transparent methacrylate plastic.

A slightly wider vertical hole was drilled in the top of the block to meet the horizontal channel, and gave access to the tip of the pressure cannula. The block was carried on the arm of a simple dissecting microscope, whose rackwork served to raise and lower it as required. The block, its support and the syringe tip of the pressure apparatus were immersed in a bath containing 30% sea water thermostatically controlled at 38–39° C. (see Fig. 1).

Continuous recording of the changes in pressure was made possible by a simple slit camera. A multi-speed Palmer kymograph was mounted with the shaft horizontal. The shaft carried a 12 in. drum enclosed in a light-tight box; in the roof of the box was a slit, $\frac{1}{2}$ mm. in width. An image of the needle of the pressure gauge was thrown on the slit by a 6 in. photographic lens used at a magnification of (×2); illumination was provided by a strip light about 50 cm. above the needle. Time marking was accomplished by a 6 V. flash-lamp bulb, mounted 50 cm. above the slit, and switched on at half-minute intervals by a time clock.

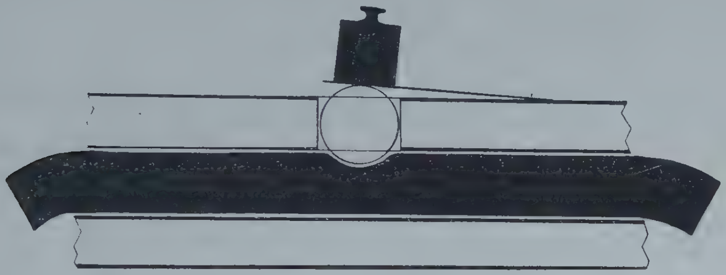

Fig. 2. A diagram of the indentation pressure gauge.

An earlier type of pressure gauge, which could be used on the intact worm, was also employed in the present series of studies. In its simplest form it is illustrated in Fig. 2. A portion of the worm was confined within a tube of 6 mm. or less in diameter, drilled longitudinally through the axis of a rectangular prism of Perspex. At right angles to the main channel, a small cylindrical hole was bored, just large enough to allow a $\frac{3}{16}$ in. steel ball bearing to move freely through it. A 2 × 1 in. cover-glass rested on the projecting surface of the ball bearing and could be loaded if required by additional weights (Fig. 2). The indentation produced by the ball depends not only on the weight of the system and on the internal pressure but also on the rigidity of the body wall, which complicates the results obtained. In practice therefore the apparatus was calibrated in the following manner. A suitable worm was cut in half; using a pair of forceps, a certain amount of the genital ducts was removed from the tail portion, which was then ligatured on to a wide glass cannula connected with a water manometer. The tail portion was threaded into the channel of the pressure gauge and the movement of the ball-bearing system was observed when different internal pressures were applied by the water manometer head. In practice the pressure required just to raise the cover-glass from the Perspex surface was determined for a range of different weights added; the calibration curve was not linear, but the method provided a simple and rapid indication of the approximate extent of the internal pressure changes in an intact and active worm.

RESULTS

(1) *Measurement of turgor pressure*

Whichever form of pressure gauge was used, its successful functioning could readily be tested by applying finger pressure to the body of the worm. The immediate and reversible response which was obtained (except when the needle was blocked) showed that there was normally free transmission of the internal pressure throughout the body. It would appear that the fluid-filled spaces in the pseudocoelomic cavity are in open communication. Occasionally, particularly when the worm had been threaded through a narrow tube, there was clear evidence of a block in transmission of pressure across the constricted region, but this has not been encountered in an unconstricted worm. The recorded pressures may therefore be taken to be the true internal turgor pressure.

Table 1. *Pressures measured by the glass helix gauge in adult female* Ascaris

Worm	Notes	Pressure	
		cm. of water	mm. Hg
A	Fresh worm	135	100
B	Three successive tests: tail region	45	33
	tail region	45	33
	vulval region (varying)	37–83	27–60
C	Three successive tests (maximum values)	142	104
		172	127
		172	127
	Same worm cooling down	30–37	22–27
	Ditto with warm saline applied to head region (two tests)	83	60
		75	55
D	Three successive tests with little variation	67–83	50–60
		67–75	50–55
		67	50
E	An active worm showing great variations in pressure (max. and min. in four tests)	15–127	11–94
		105–142	77–104
		37–135	27–100
		–150	–110
F	Worm kept in saline 4 hr. after collection	60	44
G	Ditto, another specimen	52	38
H	Ditto, 6 hr. after collection	52	38
	Same worm immediately after a defaecation	22	16
I	Worm 6 hr. after collection	37	27

Table 1 summarizes the results of a number of observations made with the glass helix pressure gauge; it illustrates the wide range of pressures encountered in different animals, as well as in the same animal at different times. The range encountered in these experiments was from 16 to 125 mm. Hg with a mean value for the fresh worms in the region of 70 mm. Hg (95 cm. of water).

The indentation pressure gauge in its present form is not suited to instantaneous determinations of internal pressure, though a modification is being designed for continuous recording. It was used on intact worms by adding a constant weight and observing the proportion of the total time during which the internal pressure was high enough to raise the cover-glass above the Perspex base. Using the values for internal pressure obtained from the method of calibration described above, it was found that the pressure only rarely fell below 25 mm. Hg, but that for 37 % of the time it was above 65 mm. Values which were above 100 mm. were found to occur for a small portion of the total time of observation.

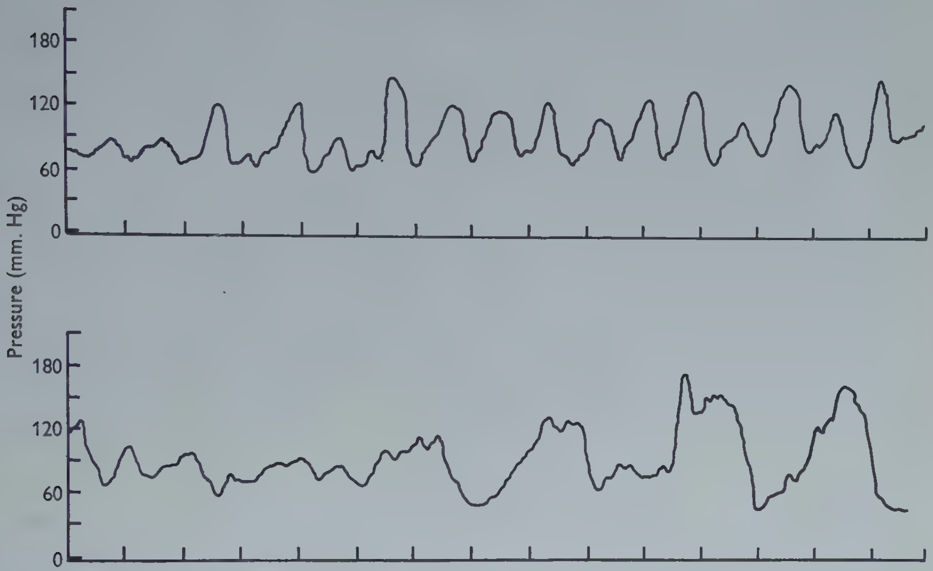

Fig. 3. Tracing from a photographic record of pressure changes made with a glass helix pressure gauge. The two halves of the record are consecutive. Time marks are at 30 sec. intervals.

The highest values which have been recorded in the present series of studies were obtained from a worm in which the head region was tightly coiled in front of the point of penetration of the cannula, and the tail was at the same time strongly contracted. For about 15 sec. the worm maintained a pressure greater than 180 mm. Hg, rising for a few seconds to a maximum of 225 mm. Hg (306 cm. of water), equivalent to nearly a third of an atmosphere.

These values are almost an order of magnitude higher than those obtained by Picken (1936) for *Potamobius fluviatilis* (10·5–18 mm. Hg), for *Carcinus moenas* (3·5–19 mm.), for *Peripatopsis* (2–15 mm.) and also than those obtained by Chapman & Newell (1947) for *Arenicola* (10–20 mm., mean values of resting and maximum pressure), by Chapman (1949) for *Calliactis parasitica* (0–10 mm.) and by Newell (1950) for *Lumbricus terrestris* (1·5–21 mm.).

That the wide variation recorded in Table 1 is a normal one is shown in the tracing of Fig. 3, taken from a photographic recording over a period of 15 min.

There is evidence of a rhythmical rise and fall in pressure at intervals of about 30 sec. in the early part of the record. The recorded pressures vary from 49 to 180 mm. Hg with a mean value of 95 mm., substantially similar to the values noted above.

Further confirmation of these high values for the internal pressure has been obtained from two other lines of experimental investigation.

The half worm, ligatured to a cannula placed in connexion with a water manometer, usually lay completely inert at low pressures—up to 15 mm. Hg. At 45 mm. occasional contractions were recorded, and from 60 to 100 mm. regular and continuous bursts of rhythmical activity occurred at intervals of about 20 sec. Above 150 mm. the activity was irregular; the tail became extended in length, and at pressures of 200 mm. and above failed to show contractions. The pressure conditions in these experiments are of course not strictly comparable with those in the intact worm, since in the isolated halves contraction is isotonic, i.e. against a constant manometer pressure; as we shall see later, contraction in the intact worm is accompanied by a rise in pressure.

As a final check on these observations, if we assume that the pressure is generated by the tension exerted by the longitudinal body musculature we can calculate its value from the experimentally observed values for this tension. Miss C. E. Bradley, working in this laboratory, has found that isolated 'anterior preparations' of *Ascaris* as used by Baldwin (1943) will show rhythmical contractions against a load of 10–20 g., but fail to do so against loads of 25–30 g. Assuming that the cross-sectional area of the preparation is 15 sq.mm. a tension of 10–20 g. corresponds to an internal pressure of 75–150 mm. Hg, which is consistent with the observed values.

(2) *The structure of the cuticle*

There is a common impression that the cuticle of nematodes is stiff and unyielding. Hyman (1951, p. 399) says that 'because of their stiff cuticle...the nematodes possess poor powers of locomotion'. Stauffer (1924), in his study on locomotion in the nematodes, says that the cuticle, in conjunction with the internal body pressure, helps to maintain a constant body length, and compares the system with the vertebral column or notochord in a vertebrate.

In actual fact the cuticle of *Ascaris* is extensible and capable of much more extension than is perhaps generally realized. We have recorded changes in length (of half worms artificially inflated as well as of the corresponding portions of intact worms) of the order of 10–15% for pressure differences within the normal physiological range; similar alterations can occur in the diameter.

In these changes in length and diameter of the body the fine structure of the cuticle appears to play an important part. Prominent among the structural components of the cuticle of *Ascaris* and of most nematodes is a set of diagonal crossed fibres, readily visible in fixed specimens. Three such fibre layers together form a spiral basketwork, incorrectly illustrated by Chitwood & Chitwood (1950).

It seems probable that the crossed fibrils described by Picken, Pryor & Swann (1947) refer to these visible fibres. They are thick and substantial compared with the other recognizable fibre systems and with the remainder of the elastic layer; it seems reasonable to regard them as practically inextensible in the living animal. Picken *et al.* have commented on the value of this arrangement in permitting anisometric extension of the cuticle.

The two sets of fibres may be considered to enclose between them a system of minute parallelograms (Fig. 4) which, even though the lengths of their sides remain constant, may be distorted by shearing with a consequent change in length of their diagonals. As these diagonals lie, in relation to the nematode, longitudinally and transversely, the distortion of the structure will lead to the worm becoming longer and thinner or shorter and thicker—the changes in length and diameter will be strictly interrelated as in a set of 'lazy tongs'.

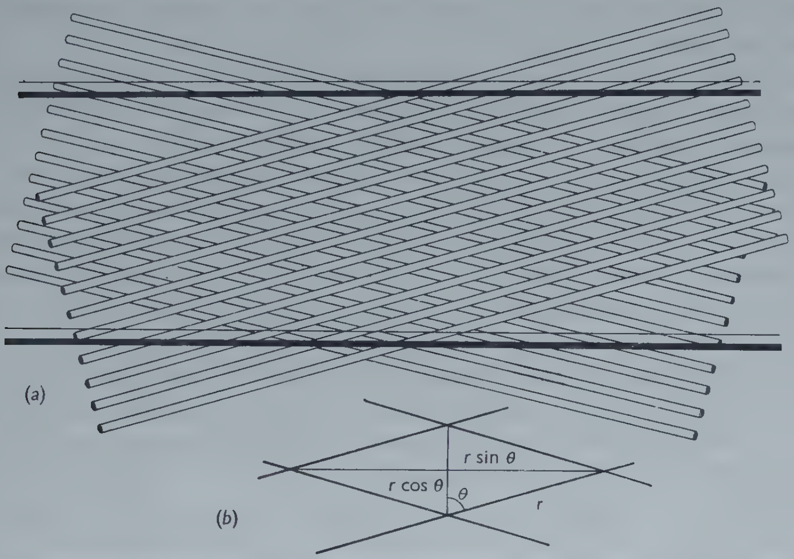

Fig. 4. A schematic representation of the basketwork structure of fibrils in the cuticle of *Ascaris*. (*a*) General diagram illustrating the orientation of two of the fibril layers in relation to two of the transverse external annulations. (*b*) The equivalent unit parallelogram.

In such a structure there will exist one critical shape of the unit parallelogram from which any small distortion will change the length, l, and diameter, a, of the worm in such a way that the volume ($\propto la^2$) remains constant.

If r is the length of any side of the parallelogram and θ is the acute angle between any side and the longitudinal axis of the worm, then

$$l \propto r\cos\theta \quad \text{and} \quad a \propto r\sin\theta,$$

so that V, the volume, $\propto r^3 \cos\theta \sin^2\theta$. The condition that V shall be unchanged by a small change in θ is provided by $dV/d\theta = 0$, i.e.

$$\frac{d}{d\theta}(\cos\theta \sin^2\theta) = 0,$$

or
$$2\sin\theta\cos^2\theta = \sin^3\theta,$$

from which
$$\tan^2\theta = 2 \quad \text{and} \quad \theta = 55^\circ.$$

If the angle θ is larger than this (i.e. if the fibres lie more nearly in the transverse plane), an increase in length will more than compensate for decrease in diameter so that the worm will increase in volume on extension; if $\theta < 55^\circ$ the reverse will be true and lengthening of the worm will decrease its volume.

Measurements on a number of fixed specimens gave a mean value for θ of $75^\circ\ 30'$, rather greater than the 70° suggested by Picken *et al.* (1947), who quoted a (possibly approximate) figure. In any case it is clear that for such an angle, length and volume changes will be directly related; if the worm increases in volume it will do so by extending and growing thinner. As it swells in this way the angle θ will decrease, but an extension of well over 50% would be required before the critical angle of 55° was reached; this extension appears to be far beyond the capabilities of the normal animal.

It was possible to make an approximate test of this hypothetical behaviour of the cuticle by measuring the change in length for a given change in volume.

A normal worm was threaded through a rubber stopper, bored with a hole sufficiently small to prevent it moving through. The stopper was thrust into a tube (containing the tail end of the worm) which was equipped with a capillary side tube to serve as a dilatometer. A series of simultaneous measurements of tail length and tail volume was made over a period in which no fluid was passing through the gut, but the worm was showing periodic tail contractions. For nineteen such contractions, the mean shortening was 8·72 mm. for a mean decrease in volume of 144·5 mm.^3. The length of the tail portion was 11 cm. and the diameter (over most of this length) 5 mm. A simple calculation (based on the assumption that the worm is a cylinder) showed that for a decrease in volume of this amount the diameter must have become larger by 0·032 mm.

It can readily be shown that for a small change in length $\frac{\delta l}{l}\frac{a}{\delta a} = \tan^2\theta$, from which $\tan\theta = 3{\cdot}323$ and $\theta = 73^\circ\ 15'$. The agreement with the observed value of $75^\circ\ 30'$ is astonishingly close, particularly when we take into account the slight shrinkage in length of the worms when they are fixed, which might well increase the value of θ in the observations on fixed specimens.

It is worth noting that the basketwork structure suggested above does not in itself provide an elastic component; a framework of this type could, however, function as an elastic structure so long as (*a*) distortion changes its volume, i.e. $\theta \neq 55^\circ$, and (*b*) it is equipped with suitable muscles. If $\theta > 55^\circ$ such muscle fibres must be longitudinal, while if $\theta < 55^\circ$ the muscle fibres must be transverse or circular.

Any increase in tonus of the longitudinal muscles, by tending to shorten the body, would also tend to reduce the volume. Since the fluid contents of the body must be considered incompressible, the volume cannot decrease and the effect of this increased tonus must be to increase the pressure in the worm. It is of course probable that, even when the muscles are completely relaxed, other elements (longitudinal and transverse fibres in, and the matrix of the cuticle itself) are capable of exerting an elastic tension against internal pressure. It may be that the lowest pressures recorded in Fig. 3 are in fact due entirely to the elastic force of the cuticle itself; increases above this resting level will then mark the activity of some part of the body musculature.

Such a system will clearly be capable of coming into static equilibrium over an appreciable range of body volume, thereby providing for the maintenance of the characteristic internal pressure (or the cycle of pressure changes) even if additional fluid is being taken into the gut, or after defaecation has released a certain volume of gut contents. Similarly, osmotic changes, which must be a normal feature of the environment of a gut parasite, can readily be dealt with if transfer of water can occur without seriously affecting the mechanical stability of the system.

In contrast to the state of affairs in a worm with an isotropic elastic cuticle, or in one in which circular and longitudinal muscle layers are present, there is in this arrangement a strict mathematical relationship between length and diameter; the internal volume will completely determine the length of the animal. At this length, the muscles can then generate pressures and provide the elasticity for the working of the system in a co-ordinated fashion. Any true elastic component of the cuticle itself will merely serve to limit the extent of increase of volume and length without otherwise affecting the argument.

A comparison of the situation in *Ascaris* with that described by Cowey (1952) in the nemertine shows an interesting and fundamental difference. So far as we have been able to discover, the internal hydrostatic pressure in an active *Ascaris* is always considerable; the cross-section of the body does not depart significantly from the circular shape and some part of the longitudinal musculature is always in a state of tonus. The angle between the fibrils and the longitudinal axis is therefore sensibly constant, as is the total length of the animal. This is a necessary consequence of the relatively free transmission of hydrostatic pressure throughout the pseudocoelomic cavity. Local shortenings in length, brought about by contraction of muscles in a particular region, are reflected in similar extensions in other parts against the elastic (or active) force exerted by the musculature in these distant regions; substantial variations in total length are found only when there are similar changes in total volume—during feeding and defaecation.

It must be realized that this picture of a basketwork with a theoretically uniform mesh over the whole of the animal is an oversimplification. It will be fairly true over a restricted length, but in a body as long as this it is clearly possible for the shape of the unit parallelogram to change gradually from one region to another. Local contraction of the tail, for example, will shorten that region by displacing fluid into the head end, where lengthening of the body will occur. The total length of the body will remain relatively unchanged, but only as a result of compensating changes in different regions of the animal. By such changes the movement of different parts of the body is largely co-ordinated, as we hope to show in a later paper; in this way a pattern of organized activity can be achieved in terms of responses which are largely local in character, i.e. which do not require an elaborate central nervous machinery of co-ordination.

It can readily be shown that changes in volume and pressure are brought about by contraction of the muscles. The graph of Fig. 5 shows that, over a period in which internal pressure and tail length were simultaneously recorded, there was a clear correlation between tail shortening and increase of pressure. In a later paper, which will be concerned with feeding and volume transfer between the head and tail regions, we hope to publish one of our records which shows (among other facts irrelevant to this present account) that volume transfer follows upon the shortening of the tail with a slight delay. This indicates that the sequence is: muscle contraction →tail shortening→decrease of tail volume and increase of pressure→volume transfer→increase of head volume and pressure→head lengthening.

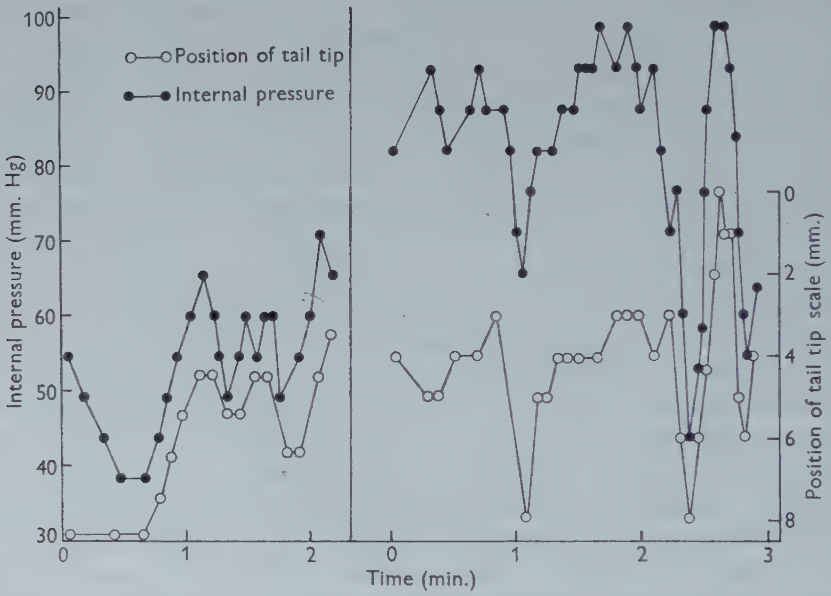

Fig. 5. The internal pressure and the position of the tail tip plotted against time for two different worms. Each worm was fixed in the region of the vulva, so that changes in the position of the tail tip represent actual changes in the length of the whole post-vulval region.

DISCUSSION AND CONCLUSIONS

In the introduction it was suggested that physical and mechanical forces determined the general body structure of nematodes and that the limitations imposed by the particular mechanical system accounted for the uniformity of the group.

In the many and varied discussions of nematode affinities (reviewed by Chitwood & Chitwood, 1950; Baylis, 1924) attempts have been made to define the essential nematode characters. Seurat (1920) defined the 'primitive nematode'; Chitwood & Chitwood (1933) listed the characters of a 'proto-nematode'; Chitwood & Chitwood (1950) described the 'primitive nema'. In all of these accounts there is a general agreement, the main differences being in emphasis and interpretation. The basic characters are as follows: the body is an elongated cylinder with a protein cuticle which is not homogeneous but has at least two component parts; of these, one is a system of inextensible diagonal or spiral fibres, and the other is elastic. The body musculature consists entirely of longitudinal fibres; the only other muscles to be found are the so-called 'sphincters' at the anus and vulva, and the specially arranged muscles in the reproductive system and in the pharynx. It is to be noted that the 'sphincters' are almost invariably 'dilators' and that the only circular muscles to be found are in the ducts of the reproductive system.

The nervous system is simple, consisting of a cephalic and caudal concentration of nerve cells linked by a few longitudinal nerve cords containing few if any nerve cells. The gut consists of a powerful pumping triradiate pharynx and a simple straight intestine composed of a single layer of cells. The body cavity is a pseudocoelom which is open and not divided serially into segments. The excretory system is basically a special system of internally closed intracellular ducts which open to the exterior. Cilia are absent; the spermatozoa are never truly flagellate and usually move in amoeboid fashion. The characteristic type of movement is by wave-like undulations of the body.

Many of the characteristics given above appear to be closely correlated with the mechanical features established by the work described in the present paper.

We have demonstrated the presence, in *Ascaris*, of a system in which the longitudinally arranged muscles act not against other antagonistic muscles but against forces exerted by the internal pressure on the cuticle. The success of this unique system depends on the presence of a spiral basketwork of inextensible fibres which permit anisometric extension and shortening of the cuticle. If body movements such as those concerned in locomotion are produced by these longitudinal muscles, it follows that the strength and efficiency of these movements can be increased only if the internal pressure of the body fluid increases also.

Such a system will be even more efficient if the cuticle contains, in addition to the main fibre network, a true elastic component. This component may be assumed to play a significant role when the meshwork is considerably extended by great elongation of the animal. Under such circumstances the elastic force will reinforce the action of the muscles, will therefore reduce the tension which must be maintained by them, and make the contraction of such muscles even more effective. This is true for the longitudinal elastic component; the circular elastic component has a corresponding role at low internal pressures, for it will, by resisting transverse expansion of the meshwork, maintain a slight longitudinal extension even when the internal pressure is negligible—keeping the muscle fibres in that state of slight stretch which is desirable for their effective action. The system is in fact designed to function not only at a high internal turgor pressure but over a wide range of pressures.

This form of organization is particularly well adapted to the production of the undulatory pattern of locomotion which is the most effective one in media of high viscosity (see Gray, 1951). At any given internal volume it provides for a constant length, against which the pull of dorsal or ventral muscles only can give a powerful bending movement of the body. It is worth noting that in such highly viscous media the presence of appendages as in *Nereis* would impede movement.

The possibility of co-ordinating the movement of different parts of the body by the transfer of fluid resulting from local changes in volume has already been referred to; with such mechanical co-ordination the need for local reflex networks does not exist. The simplicity of the nervous system is probably related to this feature.

Because of this possibility of local changes in length, the alimentary canal must be freely extensible. Such a tube will necessarily collapse under the internal pressure and can only be filled by some pumping mechanism capable of producing an even higher pressure. This is provided, as we shall show in a later paper, by the special structure of the triradiate pharynx; ciliary mechanisms would be completely incapable of generating the pressures required even if the walls of the canal were in some way distended to allow them to beat freely.

The presence of dilator muscles, e.g. the so-called 'depressor ani', is also a concomitant of the high internal pressure. The gut contents would escape, forced out by the internal pressure, if the pharynx and anus were not provided with 'self-sealing' devices requiring muscular activity only to open them.

The excretory and reproductive systems show special features equally related to the internal pressure. In the common type of excretory system found in *Ascaris* the very long excretory tubes are embedded in the lateral chords and will consequently not be collapsed by the pressure; this will in fact provide effective filtration of the excretory product even against high internal colloid osmotic pressure. In the alternative 'renette-cell' type of excretory organ the duct is free in the body cavity but very short. Here it seems likely that the cell itself is the excretory organ and, like the mammalian salivary gland, may generate pressures in its duct higher than those in the surrounding medium.

The reproductive system is the only one in the body which possesses a peristaltic musculature and true sphincter muscles. Here, however, the mechanical conditions are markedly different from those in the gut or the excretory system. There is no controllable pump like the pharynx to fill the system, and the escape of eggs in the female which would take place with a simple vulval dilator muscle would need to be limited to those which were fertilized and equipped with their shells and egg membranes. Such controlled emission can best be produced by a combination of peristaltic movement—which pinches off a selected group of ova—and a sphincter muscle (derived possibly from the same circular and oblique fibres) which releases at the appropriate stage in the peristaltic cycle. Since the ova before fertilization are amorphous and tightly packed, amoeboid spermatozoa injected at high pressure by a special male copulatory apparatus will be necessary for successful fertilization—the absence of true flagella in the sperm may, however, also be connected with the absence of cilia elsewhere.

How far are we entitled to make such sweeping generalizations about the determining factors in the structural organization of the nematodes from these special studies on *Ascaris*? There is this justification. We have shown that the mean value of the internal pressure can be calculated to a reasonably close approximation from the tension which the longitudinal muscles can exert per unit area of the body cross-section. Since this tension will itself be proportional to the cross-sectional area of the muscles, it follows that in nematodes of widely different size, if the proportion of the cross-section occupied by muscles is similar, the internal pressure will also be the same. Furthermore, since the tangential stress in a cylinder subjected to internal pressure is proportional to the diameter, the thickness of the nematode cuticle required to withstand this stress can be proportional to the diameter of the animal. The mechanical factors which determine the general features of the nematode will thus be independent of the scale on which the model is built.

SUMMARY

1. Experimental determinations of the hydrostatic pressure in the pseudocoel of living *Ascaris lumbricoides* were made by a direct method, using a glass helix pressure gauge and by an indirect method using an indentation gauge, both of which are described.

2. The mean value of this pressure was 70 mm. Hg (95 cm. of water), and showed wide and often rhythmical variations from 16 mm. to as high as 225 mm. Hg. Observations on the behaviour of artificially distended worms and of the tension developed by the muscles confirm these results.

3. The mechanical structure of the cuticle, with its inextensible spiral fibrils, forming a basketwork at an angle of 75° to the longitudinal axis, provides for an anisometric expansion and contraction under the action of the longitudinal muscles which is closely in accordance with the observed changes in volume and length.

4. A discussion of the significance of this mechanism and of the high internal pressure suggests that the great similarity of form among nematodes is determined to a considerable extent by mechanical factors.

We are glad to acknowledge the help of Mr M. Gillett in the construction of the apparatus described in the paper.

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THE FUNCTION OF THE BRAIN OF *OCTOPUS* IN TACTILE DISCRIMINATION

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INTRODUCTION

In a previous survey of the capabilities of the tactile nervous system of *Octopus* (Wells & Wells, 1956) evidence was presented suggesting that the brain correlates information from sense organs in the arms in a relatively simple manner. Animals trained to discriminate between objects differing only in their non-chemical surface characteristics could distinguish surfaces differing in degree of irregularity (e.g. by having a greater or lesser number of grooves cut into their otherwise smooth surfaces) but were unable to make discriminations dependent upon the recognition of the pattern or orientation of irregularities. It appears that octopuses do not make use of a projection of the sensory field or of any scanning mechanism in order to identify objects touched. It was suggested that these animals distinguish objects made of the same material on a basis of the proportion of mechanoreceptors stimulated in the sensory areas (the rims of the suckers) in contact, an object with a rough surface being recognized as distinct from one with a smooth surface because it stimulates a greater (or lesser) number of sensory endings, the arrangement of irregularities or the shape of the object being irrelevant. If this is correct, the ease with which it is possible to teach octopuses to discriminate between objects touched and the similarity of those objects in terms of the proportion of sensory endings that they excite should be closely correlated. The aim of the present series of experiments has been to test this correlation.

It was previously supposed (Wells & Wells, 1956) that the only way of obtaining a measure of the difficulty that an octopus has in distinguishing two objects was to count the number of trials that it took to learn to discriminate between them. This method is impractical because of the large number of experiments that it necessarily involves (see p. 134), and no attempt was made to compare the performances of individuals in this way. When further experiments were made, however, an alternative method of measuring the difficulty of discriminations was seen to be possible. It was observed that under any given set of training conditions octopuses, after an initial rapid improvement in performance, settle down to making a more or less constant proportion of errors to trials, and continue to err at this rate irrespective of the duration of training (but see p. 138). This proportion of errors is related to the similarity of the objects that they are required to distinguish, and provides a means of measuring and comparing the difficulty of different discriminations independent of the rates of learning of the experimental animals.

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MATERIAL

Octopus vulgaris Lamarck of between 250 and 1000 g. caught in the Bay of Naples were used; methods of catching and keeping these octopuses have been described by Boycott (1954). The temperature of the sea water circulating through the tanks in which the experimental animals were kept varied from 16° C. (spring and late autumn) to 23° C. (summer), the water being heated in winter to about 18° C., because at temperatures below 13° C. *Octopus* ceases to feed regularly.

Animals were trained after section of the optic nerves as described in Wells & Wells (1956). The experiments quoted are from animals trained in 1954, 1955 and 1956. Animals used in 1954 are identified by a number preceded by the prefix A (e.g. A173), those in 1955 by B, and those during 1956 by C. Experiments made with the A (1954) series of animals have been reported in a previous paper.

EXPERIMENTAL METHOD

A. *Training method*

The method used to train blind octopuses to discriminate between objects, and the movements they make in accepting or rejecting objects touched, have been described fully elsewhere (Wells & Wells, 1956). Briefly, each experiment consisted of a number of trials at each of which one or other of a pair of objects was presented to an animal, which was rewarded with a small crab or piece of sardine for passing the 'positive' object to the mouth, and given a small (6–9 V. a.c.) electric shock if it responded in the same way to the 'negative' object. There were eight trials per day, at intervals of not less than 1 hr., the objects being presented in an order that repeated itself every 8 days. As a control against initial preferences for one or other of the objects and the possibility of contamination of the positive object by food, each group of animals was divided and one-half trained to react negatively to the object positive for the other.

During these experiments animals grew very rapidly, often increasing in weight by as much as 50% during 3 weeks of training. The results with occasional animals that ceased to feed regularly and lost weight were discarded.

B. *Objects used in the tests*

A series of Perspex cylinders each 2·5 × 3·0 cm. long was used, and into all but one of them (which remained smooth) deep grooves 1 mm. wide were cut. The frequency, arrangement and orientation of the grooves relative to the length of the cylinders was varied (Fig. 1), and the pattern on the sides of the cylinders was as far as possible continued on their end surfaces, except for P2, which had ungrooved ends. It will be noted that the proportion of groove to flat surface was independent of the arrangement and orientation of the grooves. In Fig. 1 this proportion is expressed as a percentage for each object so that the similarity of any two objects can be expressed in terms of the difference between two percentages. This assumes that the pattern and orientation of the grooves is irrelevant and that *Octopus* cannot make use of these attributes in distinguishing between the members of this series of objects. This assumption is based on experiments already reported elsewhere, showing that octopuses find it difficult or impossible to distinguish between P 1 (which has grooves running along the length of the cylinder) and P 2 (grooved circumferentially) or P 6 (a pattern of squares). In the present analysis we shall show that the proportion of errors to trials made when octopuses are trained to distinguish these objects is consistent with the view that they cannot distinguish them other than by the proportion of grooves.

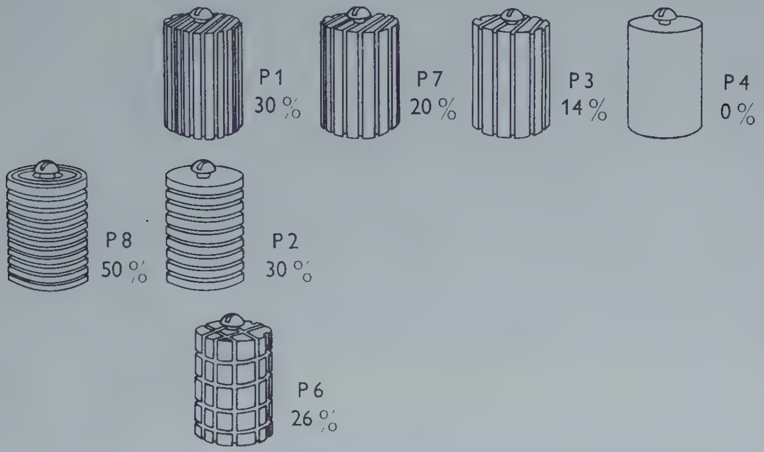

Fig. 1. The objects used in the tests. The series is arranged so that there is a decreasing proportion of grooved to flat surface from left to right. This proportion is indicated as a percentage for each object. The top row contains objects that differ only in the frequency of vertical grooves cut into them. P 2 differs from P 1 only in the orientation of the grooves cut into it. P 6 has very nearly the same proportion of groove as P 1, but the grooves are arranged to form a pattern of squares. Octopuses apparently find P 1, P 2 and P 6 difficult or impossible to distinguish, but can be taught to discriminate between the members of the top row (and between P 8 and P 4) relatively easily.

EXPERIMENTAL RESULTS

When blind octopuses in good condition are trained in the manner already described, they at first pass all the objects, positive and negative, to the mouth. Training thus consists for the most part of teaching the animals not to respond positively to the negative object, whilst retaining their positive responses to the positive object. Animals given shocks for taking the negative object learn either to withdraw the arms from contact with such objects (presented whenever possible to extended arms) or, more usually, to thrust such objects away. Responses to the positive object do not alter in nature, but increase in speed, the time taken to examine and pass this object under the web to the mouth commonly falling from about 15 sec. to less than 5 during the first twenty or thirty trials of training in simple discriminations.

There is considerable variation in the behaviour of individual octopuses during the first forty or fifty trials of training. Most animals react as described above, examining each object presented to them by feeling it over with the suckers for 5 or 10 sec., and react in a predominantly positive manner throughout training, making errors mainly by taking the negative object. A minority, after receipt of several electric shocks, become temporarily almost wholly negative in their reactions, snatching their arms away from objects touched without examining them. Such animals naturally take longer to learn to distinguish the objects in the test, although they eventually reach a condition in which their behaviour is in all respects like that of the initially more 'positive' animals. As a result of these differences in reaction to the training conditions, tending to produce a considerable scatter in the number of trials required to train animals to a given criterion, the 'number of trials to criterion' cannot be used as a means of comparing the difficulty that *Octopus* has in distinguishing the test objects under these conditions unless a very large number of experiments be made.

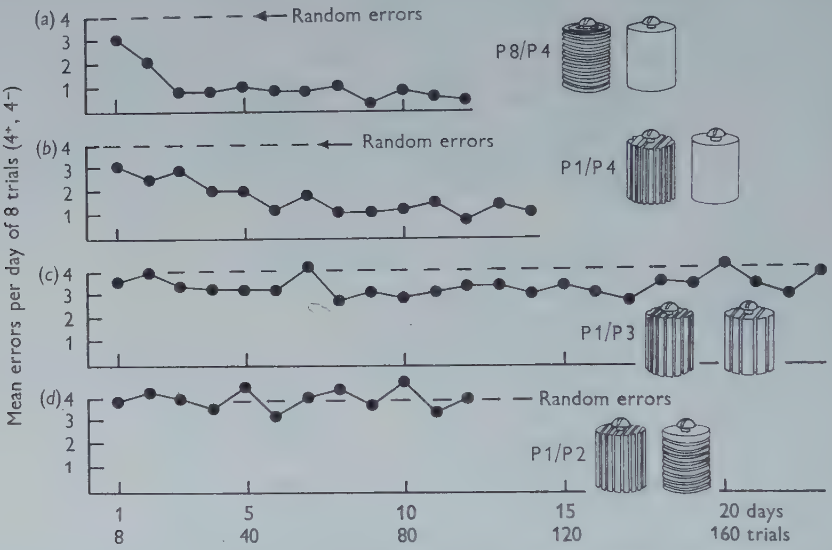

Fig. 2. A summary of the errors made in the course of four series of training experiments in each of which six (or twelve in the case of P8/P4) octopuses were trained to distinguish between two Perspex cylinders. The four series are arranged in order of difficulty for *Octopus*, P8/P4 being the easiest discrimination for them and P1/P2 the most difficult.

In Fig. 2*a* the result of training a number of octopuses to distinguish between the grooved cylinder P8 and the grooveless P4 is plotted. Evidently this is rather an easy discrimination for *Octopus* to make, and in these experiments the initial improvement in performance was rapid, so that by the end of the first day (= 8 trials, 4 positive, 4 negative) of training, typical animals made substantially less than random errors and on the third day (i.e. after only sixteen trials) better than 85 % correct responses to the test objects. This rate of improvement was not maintained, however, and from the third day onwards the daily proportion of errors to trials remained more or less constant. In similar experiments it was found that the proportion of errors made was not further reduced by increasing the voltage of the electric shocks given as punishment, despite the fact that the majority of errors were due to the animals taking the negative objects. While decreasing the voltage eventually increased the proportion of errors because the animals tended to ignore the shocks altogether, increasing them from 9 V. upwards was without effect until shocks of the order of 12–15 V. were given, at which level animals commonly became entirely negative in their reactions to the test objects, so that the proportion of total errors rose sharply, due to the animals refusing the positive objects as well as the negatives. Nor was the proportion of errors noticeably altered by variations, within wide limits, in the size or quality of the rewards given (e.g. crabs instead of fish).

It could, on the other hand, be reduced considerably by increasing the frequency of the trials. In the present series of experiments octopuses trained to distinguish P 1 from P 4 tended to settle down to making errors at a rate of about one per day of eight trials (Fig. 2*b*). In experiments to be reported in a later account octopuses were trained to discriminate between the same objects at a rate of forty trials per day; under these conditions the proportion of errors to trials dropped to about half that made under the 'eight trials per day' conditions, typical animals making about one mistake in thirteen trials as against one in eight. We have chosen to compare the performance of octopuses under training at a rate of eight trials per day because it was convenient to do experiments this way and not because there is any particular virtue in this rate of training.

Fig. 2 shows the results of training octopuses to make discriminations between the members of four different pairs of Perspex objects. These discriminations range from P 4/P 8, which octopuses can readily be trained to distinguish, to P 1/P 2, which they evidently cannot distinguish at all. It can be seen that in all cases there was very little, if any, improvement in performance after the first forty or fifty trials, the proportion of errors made thereafter remaining approximately constant even though training was continued in some cases for as many as two hundred further trials.

The proportion of errors made during any period after the first forty or fifty trials can therefore be used as a measure of the difficulty of the discrimination concerned and to predict how many errors will be made if training is continued. The ratio of errors to trials in such a period thus approximates closely to the probability of error at any one trial during that period, and the term 'probability of error' will be used here to refer to the figure so obtained, although strictly no prediction of probability can be made in training experiments unless it can be demonstrated that the animals concerned have reached a steady state.

Defined thus the probability of an individual animal erring in any given trial during the last 5 days of the P 4/P 8 series of experiments was 0·075 (a total of thirty-six errors was made by twelve animals in these forty trials). During the same period (days 8–12) of the experiments with P 4/P 1 the probability of error was 0·165, with P 1/P 3 0·39 and with P 1/P 2 0·505. It must be stressed that these figures for 'probability of error' are not directly comparable with the 'indices of correct differential response' used by Boycott & Young (1957) as a measure of the relative difficulty of visual discriminations, since the latter are based upon the errors made throughout the course of experiments including the initial period of rapid improvement.

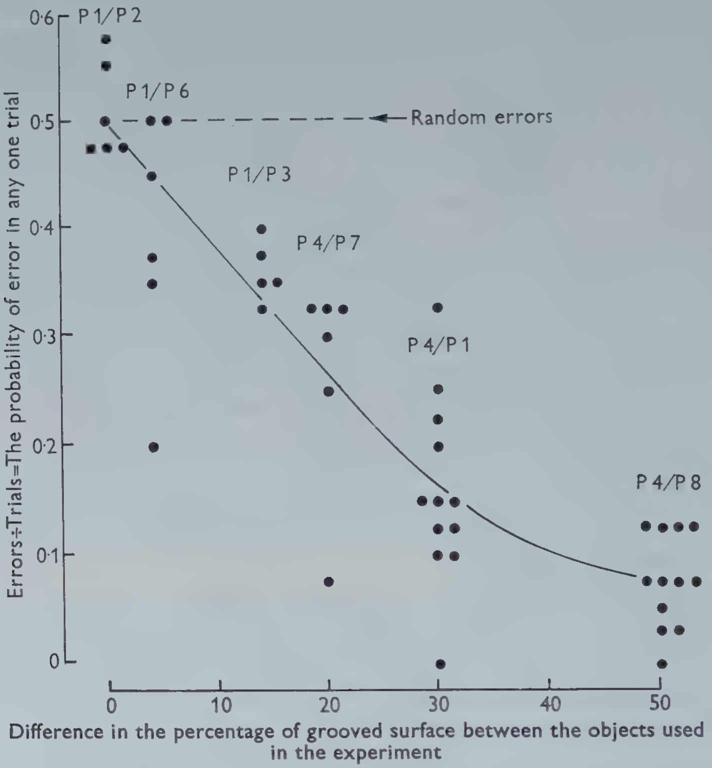

Fig. 3. Results of forty-eight training experiments, showing the relation between errors made and the similarity of objects to be discriminated. Each point shows the probability of error by one animal trained to make a stated discrimination (indicated P 1/P 2, etc.). In each case this figure is derived from the forty trials during days 8–12 of the experiment. The probabilities of error by these animals during the last forty trials of the same experiments, the length of which differed considerably, are listed in Table 1. For details of the objects used see Fig. 1.

The relation between the proportion of errors that forty-eight octopuses made in six different discriminations, and the difference between the test objects concerned expressed as the percentage of grooved surface (see description of the objects used on page 133), is plotted in Fig. 3. For this figure the proportion errors/trials was in all possible cases measured from the forty trials on days 8–12 of training (i.e. from the 56th to the 96th trial). The selection of this particular period was determined by the P8/P4 experiments, which ended after 12 days. Table 1 shows the total errors made during this period by each individual octopus, together with the total errors made by the same animal during the *last* forty trials of its training. It can be seen

Table 1

Discrimination	Animal	Errors in 40 trials on days 8–12 of exp.	Errors in last 40 trials	Probability of error in last 40 trials	Total length of exp. in trials at 8 per day	Previous exps.
P8/P4	C23	5	5	0·125	96	P1/P3
	C26	5	5	0·125	96	P1/P2
	C27	0	0	0·0	96	P1/P3
	C28	3	3	0·075	96	P1/P2
	C34	5	5	0·125	88	P1/P2
	C96	1	1	0·025	96	—
	C113	3	3	0·075	96	—
	C114	1	1	0·025	96	—
	C124	3	3	0·075	96	—
	C127	5	5	0·125	96	—
	C132	2	2	0·05	96	—
	C135	3	3	0·075	96	—
P1/P4	A127	10	10	0·25	72	—
	A133	6	6	0·15	72	—
	A135	4	4	0·1	72	—
	A159*	13	13	0·325	112	P1/P3
	A166*	8	10	0·25	112	P1/P6, P3/P5
	A173*	4	4	0·1	112	P1/P6
	A185*	6	5	0·125	128	P1/P6
	A186*	5	4	0·1	112	P1/P6
	A194	9	9	0·225	72	P1/P2
	B10*	0	0	0·0	120	—
	B42	5	5	0·125	96	—
	B43	6	6	0·15	88	—
P7/P4	B178	3†	2	0·05	184	—
	B180	13†	4	0·1	184	—
	B181	10†	10	0·25	184	—
	B183	13†	15	0·375	136	—
	B186	13†	15	0·375	144	—
	B187	12†	10	0·25	144	—
P1/P3	A159	15	17	0·425	240	—
	A168	22	23	0·575	240	—
	C4	13	18	0·45	192	—
	C5	14	14	0·35	192	—
	C23	16	16	0·4	192	—
	C27	14	17	0·425	192	—
P1/P6	A158	15	20	0·5	184	P3/P5
	A160	8	8	0·2	88	P3/P5
	A166	18	21	0·525	160	P3/P5
	A173	20	19	0·475	192	—
	A185	20	19	0·475	192	—
	A186	14	17	0·425	192	—
P1/P2	A194	23	18	0·45	160	—
	A203	20	20	0·5	88	—
	C26	22	21	0·525	144	—
	C28	19	20	0·5	144	—
	C29	19	19	0·475	104	—
	C30	19	19	0·475	144	—
P1/P5	A145	5	5	0·125	56	—
P3/P5	A145	6	8	0·2	144	P1/P5
	A158	12	11	0·275	144	—
	A160	11	11	0·275	144	—
	A166	15	19	0·475	144	—

* Animals used for plotting Fig. 2*b*.

† From days 12 to 16, experiments done in winter and heating broke down during days 8 to 12.

In experiments lasting less than 12 days (=96 trials) figures given in the 'Errors on days 8–12' column are from the last forty trials in each case.

that continuation of training after the first fifty trials (up to 200 trials in the case of difficult discriminations such as P 1/P6 or P 1/P3) rarely led to any further improvement in performance. In fact training under these conditions appears to increase rather than decrease the probability of error when continued for more than 100 or so trials. Of twelve animals trained to discriminate P 1 from P6 or P 1 from P3, seven made a higher proportion of errors in the last forty trials of training after an average of 144 trials, than in the forty trials on days 8–12 after only fifty-six trials; of the remaining five, three made exactly the same proportion of errors and only two showed a slight improvement in performance.

Why long continuation of training should lead to an increase in the proportion of errors made is obscure. It is possible that the octopuses become accustomed to receiving electric shocks; it is also possible that the rewards, remaining the same size throughout the experiment, become inadequate as the animals grow bigger and presumably hungrier, although it is unlikely that they were fed to anything approaching repletion at any time during these experiments. A similar tendency for the total errors to increase when training is long continued can be traced in visual discrimination experiments (Sutherland, 1957).

The spread of results obtained when a number of octopuses are trained to make the same discrimination is due to individual variation and is not an artifact produced by defects in the experimental method. It can be seen in Table 1 that with few exceptions animals making a relatively small number of errors in one discrimination performed correspondingly well in the next, and vice versa. The wide individual differences in ability, particularly evident in the case of very difficult discriminations such as P1/P6, are presumably due to using animals from a wild population. *Octopus* is a solitary animal, each individual living, so far as is known, in a rather restricted territory around its home, so that, in addition to the genetical source of variation, it is likely that the behaviour of animals will differ widely due to their individual experiences in the sea before capture.

Results with another Perspex object

In the original series there was an object P5. This was like the others in size and shape, but the grooves cut into it were 2 mm. instead of 1 mm. wide. P5 had eleven such vertical grooves and therefore a proportion of grooved to flat surface similar to P1 (P5 28%, P1 30%). In the case of the objects other than P5 the percentage of grooved surface can be taken as a measure of the proportion of the surface of the applied suckers *not* in contact with the object. In the case of P5 this measurement was seen to be meaningless when it was observed that the rims to the suckers of most of the experimental animals were flexible enough to touch the bottom of the wide grooves on this object.

In so far as the proportion of the area of the suckers in contact was concerned, P5 therefore more closely resembled P4, which was entirely smooth, than it did any of the other objects. This is reflected in the experimental results. An animal (A 145) trained to discriminate between P1 and P5, which on a basis of groove proportion should have been almost impossible for it, made a proportion of errors comparable with that made by other animals in distinguishing P1 from P4 (five errors in forty trials against an average of 6·3 errors made by animals in P1/P4 discriminations). When the same animal and three others were trained to discriminate between P3 and P5 the average number of errors made was eleven. Although no P3/P4 experiments were made, the average number of errors in P4/P7 discriminations (P7 being the object most like P3 in the present series) was 10·6; one would expect the proportion of errors in P3/P4 experiments to be slightly higher than this because P3 is more like P4 than is P7 (its surface being 16% grooves against P7's 20%). When this is taken into account it can be seen that the figure of eleven for the P3/P5 experiments is reasonable if P5≡P4 or nearly so.

Whilst these results confirm the view that the experimental octopuses were distinguishing the objects on a basis of the proportion of the sucker surface in contact with them, they have not been included in the summary in Fig. 3 because of the impossibility of calculating the correct proportion of groove (untouchable) to smooth (touchable) surface on P5.

DISCUSSION

It is relatively easy to teach octopuses to distinguish between Perspex cylinders that differ in having a greater or lesser number of grooves cut into them, but difficult or impossible to teach them to make discriminations dependent upon the recognition of the arrangement of these grooves relative to one another or to the shape of the cylinders. The proportion of errors made in discriminating between any two such cylinders is closely related to the difference in percentage of groove on their surfaces, the probability of error and the groove percentage difference being related almost linearly over the range 0–30% (Fig. 3). This is equally true of discriminations such as P1/P3, P1/P4, in which the objects differ only in the frequency of grooves cut into them and of others (P1/P2, P1/P6) where the objects are also potentially separable using different criteria. Evidently octopuses do not make use of cues such as the orientation or pattern of irregularities in distinguishing these cylinders. The groove percentage difference can therefore be taken as a measure of the similarity of these objects to *Octopus*, and used to predict the proportion of errors that will be made in further discriminations of this type.

That *Octopus* does not make use of the arrangement of surface irregularities relative to each other or to the shape of objects can probably be attributed to the extreme mobility of its suckers. It seems probable that the brain of *Octopus* is not capable of integrating proprioceptive information about the relative position of its individual suckers with tactile information from the sense organs on these in sufficient detail to permit the recognition of patterns of irregularity on surfaces touched (Wells & Wells, 1956).

The tactile system of *Octopus* does nevertheless permit it to recognize quite small differences in the percentage of irregularities on surfaces touched, and this is seen to be explicable when the structure of the relevant parts of the sensory nervous system in the arms is examined. The sensory endings in the suckers are of at least two types.

Of these it seems likely that the fusiform endings, which are found throughout the skin and are most common in the rims of the suckers, are the mechanoreceptors concerned in distinguishing objects touched. Alexandrowicz (1927) has described such endings in detail from the cornea and orbital membrane of the eye of *Sepia* where they probably act as receptors recording distortions of the eyeball produced by pressure changes arising from accommodation or sudden movement (Alexandrowicz, 1928). He also found similar endings in the skin of the arms of *Sepia*, and we have ourselves found a great many endings of similar appearance in the suckers of *Octopus*. There are several thousand such receptors in each sucker, distributed more or less irregularly but particularly in the rims of the suckers which are more sensitive to mechanical stimulation than any other part of the arms (Ten Cate, 1928). These endings have nerves running from them that join with the sensory nerves described by Guérin (1908) as coming from areas in and around the rims of the suckers. Each sucker is represented by a ganglionic swelling in the axial nerve cord above it, and the sensory nerves from the receptors in the suckers run directly into that ganglion where they ramify in the central white matter of the nerve cord. Physiological evidence (von Uexküll, 1894) suggests that some pass into the neighbouring two or three ganglia but for no greater distance along the arms.

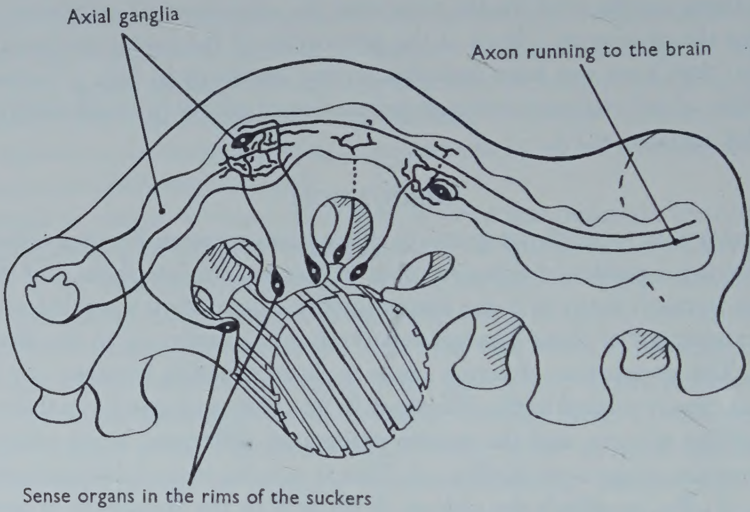
Axial ganglia
Axon running to the brain
Sense organs in the rims of the suckers

Fig. 4. Diagram of part of an arm of an octopus touching one of the test objects (P 1). Only sensory nerves are shown. The existence of pathways from the sense organs passing through the axial ganglia, and of long axons from the axial ganglia to the brain is inferred from physiological data and degeneration experiments respectively (see text).

In the axial ganglia there are nerve-cell bodies that have processes running along the arms towards the brain. Section of an arm at a point about half-way along its length produces degenerating fibres among the nerves entering the brain from the arms, so that at least some of these processes run directly into the brain. The situation is summarized in Fig. 4.

Such a system would have the properties already described provided that the brain is capable of frequency analysis and the individual mechanoreceptors discharge or alter a resting rate of discharge when stimulated. Whether the sense organs discharge when in contact with a surface (the grooves in the test objects representing areas in which they are not excited) or when distorted (by bulging of the rims of the suckers into the grooves) is irrelevant. In either case the number of nerve impulses arriving in the ganglion over a sucker will be a reflexion of the degree of irregularity of the surface of any object touched by that sucker.

One effect of any increase or decrease in the number of nerve impulses arriving in an axial ganglion will be to change the general level of excitation and therefore the state of the individual cell bodies within that ganglion. As has already been pointed out, a number of these cell bodies have axons running directly to the brain. The frequency of nerve impulses along these axons is presumably related to the state of excitation within the ganglia in which they arise and thus represents the information from many individual sense organs in a summarized form.

The brain of *Octopus* must therefore be capable of setting in motion reactions whose nature (acceptance or rejection) is dependent upon quantitative differences in the sum of nerve impulses reaching it as a result of contact with objects encountered by the arms. There is no need to suppose that it can gain additional information about such objects by simultaneous or successive comparisons of the inputs from different nerve fibres as would be necessarily involved, for example, in the recognition of patterns of irregularity.

SUMMARY

The results of fifty-three experiments in which octopuses were trained to make tactile discriminations between the members of pairs of Perspex cylinders are reported. Grooves cut into these otherwise smooth cylinders varied in number and arrangement. The proportion of errors made in distinguishing such objects depends upon the difference between the proportions of groove on the objects concerned, and is not affected by the pattern or orientation of the grooves. It has thus been possible to measure the similarity to *Octopus* of the objects used and to predict the errors that will be made in any such discrimination.

When these results are considered in the light of the known nervous arrangements in the arms, it is possible to present a hypothesis about the mode of action of the peripheral tactile sensory system and the function of the brain. It is necessary to suppose that the latter distinguishes frequencies of nerve impulses in the sensory nerves from the arms; it is not necessary to postulate any projection of the sensory field or scanning mechanism involving the use of proprioceptive information.

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